BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number
WO 01/24637 A1

(51) International Patent Classification[7]: A01N 65/00

(21) International Application Number: PCT/US00/27391

(22) International Filing Date: 4 October 2000 (04.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/157,621 4 October 1999 (04.10.1999) US

(71) Applicants: VION PHARMACEUTICALS, INC. [US/US]; Four Science Park, New Haven, CT 06511 (US). YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06520 (US).

(72) Inventors: BERMUDES, David, G.; 524 North Main Street, Wallingford, CT 06492 (US). LOW, Kenneth, Brooks; 1211 West Lake Avenue, Guilford, CT 06437 (US). PAWELEK, John, M.; 154 Woodlawn, Hamden, CT 06517 (US).

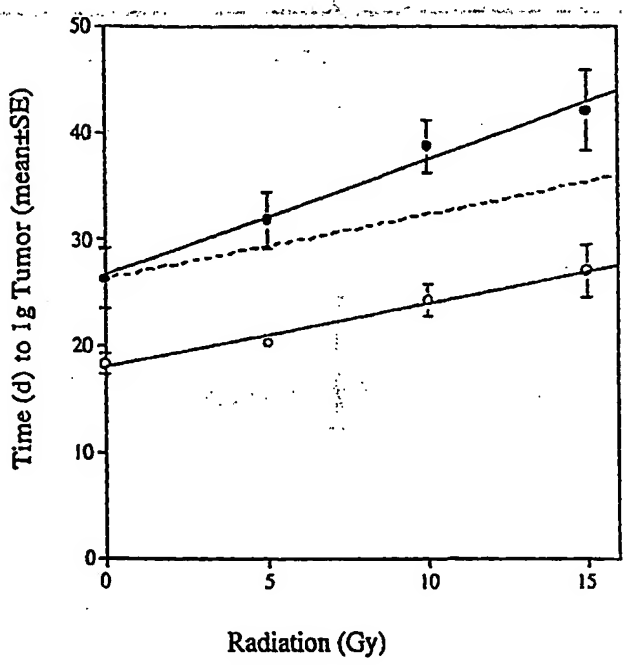
(74) Agents: BALDWIN, Geraldine, F. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,

[Continued on next page]

(54) Title: METHODS FOR TREATING SOLID TUMORS WITH IRRADIATION AND BACTERIA



(57) Abstract: The present invention provides a combination treatment method for reducing the volume of, or inhibiting the growth of, a sarcoma, carcinoma, or other solid tumor cancer, in a subject by administering to the subject in need of such treatment, an effective dose of irradiation and an effective amount of tumor-targeted facultative aerobic or facultative anaerobic bacteria. In one embodiment, the tumor-targeted bacteria are super-infective. In another embodiment, the bacteria have been genetically engineered to express a gene product(s) of interest and serve as a bacterial vector which gene product aids in reducing the volume of,

[Continued on next page]

WO 01/24637 A1

IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *With international search report.*

— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

or inhibiting the growth of, the solid tumor cancer. The gene product of interest is selected from the group consisting of proteinaceous and nucleic acid molecules. In a preferred embodiment, the bacteria or bacterial vector is attenuated. In another preferred embodiment, the bacterial vector is a tumor-targeted, attenuated vector expressing a gene product of interest which aids in reducing the volume, or inhibiting the growth of the tumor. The present invention is based, in part, on the surprising discovery that a combination method of administering one or more doses of irradiation and tumor-targeted facultative aerobic or facultative anaerobic bacteria results in greater tumor volume reduction and tumor growth inhibition than would be expected. The bacteria can also be genetically engineered to be more responsive to DNA damaging agents, such as ionizing radiation. The combination methods of the present invention give an enhanced effect, *i.e.*, more than the additive effect expected following administration of tumor-targeted bacteria and irradiation together.

METHODS FOR TREATING SOLID TUMORS WITH IRRADIATION AND BACTERIA

The present application claims priority to U.S. Provisional Application No. 60/157,621, filed October 4, 1999, the disclosure of which is incorporated by reference herein in its entirety.

1. FIELD OF THE INVENTION

The present invention relates to a combination treatment method for reducing the volume of, or inhibiting the growth of, a sarcoma, carcinoma, or other solid tumor cancer, in a subject by administering to the subject in need of such treatment, an effective dose of irradiation and an effective amount of tumor-targeted facultative aerobic or facultative anaerobic bacteria. The bacteria can also be genetically engineered to express a gene product which aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer. The bacteria can also be genetically engineered to be more responsive to DNA damaging agents, such as ionizing radiation. The combination methods of the present invention give an enhanced anti-tumor effect, *i.e.*, more than the additive effect expected following administration of tumor-targeted bacteria and irradiation together.

2. BACKGROUND OF THE INVENTION

2.1 CANCER CHEMOTHERAPY

A major problem in the chemotherapy of solid tumor cancers is delivery of therapeutic agents, such as drugs, in sufficient concentrations to eradicate tumor cells while at the same time minimizing damage to normal cells. Thus, studies in many laboratories are directed toward the design of biological delivery systems, such as antibodies, cytokines, and viruses for targeted delivery of drugs, pro-drug converting enzymes, and/or genes into tumor cells. *See, e.g.*, Crystal, R.G., 1995, Science 270:404-410.

2.2 BACTERIAL INFECTIONS AND CANCER

Early clinical observations reported cases in which certain cancers were reported to regress in patients with bacterial infections. See, Nauts *et al.*, 1953, Acta Medica. Scandinavica 145:1-102, (Suppl. 276); Shear, 1950, J.A.M.A. 142:383-390. Since these observations, Lee *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:1847-1851 (Lee *et al.*) and Jones *et al.*, 1992, Infect. Immun. 60:2475-2480 (Jones *et al.*) isolated mutants of *Salmonella typhimurium* that were able to invade HEp-2 (human epidermoid carcinoma) cells *in vitro* in significantly greater numbers than the wild type strain. The "hyperinvasive"

mutants were isolated under conditions of aerobic growth of the bacteria that normally repress the ability of wild type strains to invade HEp-2 animal cells. However, such hyperinvasive *Salmonella typhimurium* as described by Lee *et al.* and Jones *et al.* carry the risk of pan-invasive infection and could lead to wide-spread bacterial infection in the cancer patient.

Carswell *et al.*, 1975, Proc. Natl. Acad. Sci. USA 72:3666-3669, demonstrated that mice injected with bacillus Calmette-Guerin (BCG) have increased serum levels of TNF and that TNF-positive serum caused necrosis of the sarcoma Meth A and other transplanted tumors in mice. As a result of such observations, immunization of cancer patients with BCG injections is currently utilized in some cancer therapy protocols. See Sosnowski, 1994, Compr. Ther. 20:695-701; Barth and Morton, 1995, Cancer 75 (Suppl. 2):726-734; Friberg, 1993, Med. Oncol. Tumor. Pharmacother. 10:31-36 for reviews of BCG therapy.

However, TNFα-mediated septic shock is among the primary concerns associated with bacteria, and can have toxic or lethal consequences for the host (Bone, 1992, JAMA 268: 3452-3455; Dinarello *et al.*, 1993, JAMA 269: 1829-1835). Further, dose-limiting, systemic toxicity of TNFα has been the major barrier to effective clinical use. Modifications which reduce this form of an immune response would be useful because TNFα levels would not be toxic, and a more effective concentration and/or duration of the therapeutic vector could be used.

2.3 TUMOR-TARGETED BACTERIA

Genetically engineered *Salmonella* have been demonstrated to be capable of being tumor-targeted, possess anti-tumor activity and are useful in delivering genes such as the herpes simplex thymidine kinase (HSV tk) to solid tumors (Pawelek *et al.*, WO 96/40238).

2.4 DECREASED INDUCTION OF TNFα BY MODIFIED BACTERIAL LIPID A

Modifications to the lipid composition of tumor-targeted bacteria which alter the immune response as a result of decreased induction of TNFα production were suggested by Pawelek *et al.* (Pawelek *et al.*, WO 96/40238). Pawelek *et al.* provided methods for isolation of genes from *Rhodobacter* responsible for monophosphoryl lipid A (MLA) production. MLA acts as an antagonist to septic shock. Pawelek *et al.* also suggested the use of genetic modifications in the lipid A biosynthetic pathway, including the mutation *firA*, which codes for the third enzyme UDP-3-O (R-30 hydroxylmyristoly)-glucosamine N-acyltransferase in lipid A biosynthesis (Kelley *et al.*, 1993, J. Biol. Chem. 268: 19866-

19874). Pawelek *et al.* showed that mutations in the *firA* gene induce lower levels of TNFα. In *Escherichia coli*, the gene *msbB* (*mlt*) which is responsible for the terminal myristalization of lipid A has been identified (Engel, *et al.*, 1992, J. Bacteriol. 174:6394-6403; Karow and Georgopoulos 1992, J. Bacteriol. 174: 702-710; Somerville *et al.*, 1996, J. Clin. Invest. 97: 359-365). Genetic disruption of this gene results in a stable non-conditional mutation which lowers TNFα induction (Somerville *et al.*, 1996, J. Clin. Invest. 97: 359-365; Somerville, WO 97/25061).

Hone and Powell, WO97/18837 ("Hone and Powell"), disclose methods to produce gram-negative bacteria having non-pyrogenic Lipid A or LPS. Hone and Powell propose using non-pyrogenic bacteria only for vaccine purposes.

Maskell, WO98/33923, describes a mutant strain of *Salmonella* having a mutation in the *msbB* gene which induces TNFα at a lower level as compared to a wild type strain.

Bermudes *et al.*, WO 99/13053, teach compositions and methods for the genetic disruption of the *msbB* gene in tumor-targeted *Salmonella*, which result in *Salmonella* possessing a lesser ability to elicit TNFα and reduced virulence compared to the wild type. In certain embodiments, some such mutant *Salmonella* have increased sensitivity to chelating agents as compared to wild type *Salmonella*. In other embodiments, the mutant tumor-targeted *Salmonella* deliver a gene product such as a pro-drug converting enzyme useful as an anti-tumor agent.

2.5 ANAEROBIC BACTERIA, X-RAYS AND CANCER

Gericke *et al.*, 1979, Journal of Microwave Power 14(2):163-166 and Gericke *et al.*, 1979, Zbl. Bakt. Hyg., I. Abt. Orig. A 243:102-112 each describe the treatment of Harding-Passey melanoma in mice using a combination regimen of local X-irradiation, high-frequency hyperthermia, and administration of clostridial spores, *i.e.*, of the obligate anaerobe *Clostridium oncolyticum s. butyricum*.

Minton *et al.*, 1995, FEMS Microbiology Reviews 17:357-364 describe the use of *Clostridium* spores, *Clostridium* being an obligate anaerobe, in combination with other agents, including X-ray irradiation, to treat solid tumors having necrotic areas. (See, Minton *et al.* at page 358 and references cited therein).

None of these references discuss the use of tumor-targeted, facultative aerobic or facultative anaerobic bacteria or bacterial vectors, in combination with other agents, including X-ray irradiation, to treat solid tumors. Further, these references do not discuss the use of facultative aerobic or facultative anaerobic bacterial vectors for delivery of therapeutic proteins to solid tumor sites.

2.6 KILLED BACTERIA, X-RAYS AND CANCER

Several references discuss the combination of X-irradiation and administration of killed bacteria for treatment of tumors. For example, Suit *et al.*, 1976, Cancer Res. 36:1305-1314 describe the administration of formalin killed *Corynebacterium parvum* to mice with a methylcholanthrene-induced fibrosarcoma in combination with X-irradiation localized to the tumor area. Suit *et al.* found that administration of the killed bacteria before local irradiation improved the results of low-level irradiation but only slightly affected the anti-tumor response at higher doses of radiation (page 1312, left column). See also, Suit *et al.*, 1977, Cancer Res. 37:4233-4234; Suit *et al.*, 1975, Nature 255:493-4494; Suit *et al.*, 1977, Cancer Res. 37:3869-3875; Suit *et al.*, 1976, Cancer 37:2573-2579; Choi *et al.*, 1979, Europ. J. Cancer 15:433-442; Collins and Wong, 1977, Radiology 125:235-241.

Ullrich and Adams, 1978, Radiation Res. 73:267-273 describe the administration of formalin killed *Corynebacterium parvum* to mice with a murine line 1 lung carcinoma in combination with X-irradiation localized to the tumor area. Ullrich and Adams found that *Corynebacterium parvum* administration was more effective in facilitating local control of the tumor and inhibiting metastatic spread when given after radiation exposure than before (page 267, Abstract).

The authors of the above-references do not discuss the use of live tumor-targeted, facultative aerobic or facultative anaerobic bacteria or bacterial vectors, alone or in combination with any other agent, including X-ray irradiation, to treat solid tumors, nor do they discuss the use of live facultative aerobic or facultative anaerobic bacterial vectors for delivery of therapeutic proteins to solid tumor sites.

2.7 BACTERIAL DELIVERY OF AN AGENT

Carrier *et al.*, 1992, J. Immunology 148:1176-1181 describe intravenous administration of an auxotrophic mutant *Salmonella* vaccine strain, which naturally infects the liver and spleen, expressing human IL-1β into normal, non-tumor bearing mice and expression of the IL-1β as measured by an antibody response against IL-1β. Moreover, expression was evidenced by that fact that mice administered the IL-β expressing *Salmonella* were protected from a lethal dose of total body γ-irradiation. Carrier *et al.* conclude that such vaccine strains of *Salmonella* can be used to express proteins *in vivo*. In addition, Carrier *et al.* indicate that non-recombinant *Salmonella* provided some protection to mice receiving a lethal dose of total body γ-irradiation. There is, however, no suggestion in Carrier *et al.* of tumor-targeted strains of *Salmonella*. There is also no suggestion in Carrier *et al.* to use irradiation in combination with administration of *Salmonella* as a

treatment for any condition, much less for their use in combination with irradiation to treat solid tumors.

Citation or identification of any reference in Section 2, or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides a combination treatment method for reducing the volume of, or inhibiting the growth of, a sarcoma, carcinoma, or other solid tumor cancer, in a subject by administering to the subject in need of such treatment, an effective dose of irradiation and an effective amount of tumor-targeted facultative aerobic or facultative anaerobic bacteria. In one embodiment, the tumor-targeted bacteria are super-infective. In another embodiment, the bacteria have been genetically engineered to express a gene product(s) of interest which gene product aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer. In a preferred embodiment, the bacteria are attenuated. In another preferred embodiment, the bacteria are tumor-targeted, attenuated bacteria expressing a gene product of interest which aids in reducing the volume, or inhibiting the growth of the tumor. In yet another embodiment, the bacteria are genetically engineered to be more responsive to a DNA damaging agent, such as ionizing irradiation, oxygen free radicals, ultraviolet light, etc. In yet another preferred embodiment, the bacteria are attenuated, tumor-targeted, express a gene product of interest, and are genetically modified to be more responsive to a DNA damaging agent. In yet another preferred embodiment, the dose of irradiation is localized to the tumor site.

The present invention is based, in part, on the surprising discovery that a combination method of administering one or more doses of irradiation and tumor-targeted facultative aerobic or facultative anaerobic bacteria results in greater tumor volume reduction and tumor growth inhibition than would be expected. The combination methods of the present invention give an enhanced effect, *i.e.*, more than the additive effect expected following administration of tumor-targeted bacteria and irradiation together.

Facultative aerobic and facultative anaerobic bacteria useful for the methods of the present invention include but are not limited to *Escherichia coli*, including but not limited to pathogenic, including uropathogenic *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis*. In a preferred embodiment, the bacteria are an attenuated strain of *Salmonella* genetically engineered to express an altered Lipid A which reduces the virulence of the strain, and genetically engineered to express a gene product which aids in preventing the

growth of a solid tumor, which gene product is under the control of an irradiation-inducible promoter. In yet another preferred embodiment, the bacteria are genetically engineered to be more responsive to a DNA damaging agent, *e.g.*, increased inducibility of SOS-type promoters. Such increased responsiveness, *e.g.*, is the result of a mutation at a locus involved in DNA recombination or repair, such as *recN*. Such increased responsiveness can also result from a mutation at a locus involved in the prevention of DNA damage through the destruction of free radicals, *e.g.*, *oxyR* or *soxR*. As used herein, more responsive is at least 5% more responsive than the corresponding strain without the mutation. Thus, in certain embodiments, the mutated strain is at least 5%, at least 10%, at least 15%, at least 25%, at least 30%, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%, or at least 100% more responsive to a DNA damaging agent than the corresponding non-mutated strain.

The gene product of interest is selected from the group consisting of proteinaceous and nucleic acid molecules. In various embodiments, the proteinaceous molecule is a cellular toxin (cytotoxic agent), *e.g.*, saporin, cytotoxic necrotic factor-1 or cytotoxic necrotic factor-2, a ribosome inactivating protein, or a porin protein, such as gonococcal PI porin protein. In other embodiments, the proteinaceous molecule is an anti-angiogenesis protein or an antibody. In yet other embodiments, the proteinaceous molecule is a cytokine, *e.g.*, IL-2, or a pro-drug converting enzyme, *e.g.*, Herpes Simplex Virus ("HSV") thymidine kinase or cytosine deaminase. The nucleic acid molecule can be double stranded or single stranded DNA or double stranded or single stranded RNA, or a triplex nucleic acid molecule. The nucleic acid molecule can function as a ribozyme, DNazyme or antisense nucleic acid, etc.

Illustrative examples of sarcomas, carcinomas, or other solid tumor cancers include, but are not limited to, germ line tumors, tumors of the central nervous system, breast cancer, prostate cancer, cervical cancer, renal cancer, bladder cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, mesoendothelioma, mesothelioma, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma.

The source of the irradiation can be gamma rays or X-rays. The treatment may comprise a single dose of irradiation or may comprise several doses of irradiation (fractionated doses). The effective dose of irradiation can be calculated using methods known in the art taking into account the overall health of the patient and the type and location of the solid tumor. An illustrative example of a course of radiation treatment for a human patient with a solid tumor is local administration of irradiation to the tumor site of 2 Gy/day for 5 days per week for 6 weeks (total exposure of 60 Gy).

The present invention is also directed to a method for screening to identify bacteria that are more responsive to a DNA damaging agent using the halo assay described by Pugsley and Oudega, 1987, "Methods for Studying Colicins and Their Plasmids", pp. 105-161, In Plasmids, a Practical Approach, K.G. Hardy (ed.), IRL Press, Oxford, UK; and by Gilson et al., 1990, EMBO J. 9:3875-3884. In one embodiment, the method comprises mutating a bacterial culture containing a plasmid coding for the colicin E3 toxin under the control of a SOS-type promoter to produce a mutated bacterial culture, plating an individual colony clone of the mutated bacterial culture on to a bacterial culture plate containing a lawn of E3 toxin sensitive bacteria, culture for a time sufficient for background expression of the E3 toxin, measuring a background clearing zone (halo formation) around the colony, exposing the colony to a DNA damaging agent to induce expression of the E3 toxin, culturing the bacterial culture for a time sufficient for induced expression of the E3 toxin, and measuring the induced clearing zone (halo formation) around the colony. Those bacterial cultures having larger clearing zones are more responsive to the DNA damaging agent. In certain aspects of this embodiment, approximately 300 colony clones are plated. In yet other aspects, image analysis is used to measure halo formation.

In an alternative embodiment, the method comprises mutating a bacterial culture containing a plasmid coding for the colicin E3 toxin under the control of a SOS-type promoter to produce a mutated bacterial culture, plating an individual colony clone of the mutated bacterial culture on to a bacterial culture plate, culturing for a time sufficient for colony formation, transferring a colony to liquid culture, exposing the liquid culture to a DNA damaging agent, culturing for a time sufficient for induced expression of the E3 toxin, diluting the liquid culture, plating the diluted culture on a bacterial culture plate containing a lawn of E3 toxin sensitive bacteria, culturing for a time sufficient for the E3 toxin to detectably kill the E3 sensitive bacteria, and measuring a clearing zone (halo) around the plated bacterial culture. Those bacterial cultures having larger clearing zones are more responsive to the DNA damaging agent. In one aspect of this embodiment, the liquid bacterial culture is centrifuged and the bacteria-free supernatant is plated on the bacterial culture plate. In another aspect of this embodiment, the culture is diluted by at least a factor of 100.

3.1 DEFINITIONS

As used herein, *Salmonella spp.* encompasses all *Salmonella* species, including: *Salmonella typhi*, *Salmonella choleraesuis*, and *Salmonella enteritidis*. Serotypes of *Salmonella* are also encompassed herein, for example, *typhimurium*, a subgroup of *Salmonella enteritidis*, commonly referred to as *Salmonella typhimurium*.

Anti-angiogenic factor: An anti-angiogenic factor is any proteinaceous molecule which has anti-angiogenic activity, or a nucleic acid encoding such a proteinaceous molecule. In a preferred embodiment, the anti-angiogenic factor is a peptide fragment or cleavage fragment of a larger protein.

Attenuation: Attenuation is a modification so that a bacterium or bacterial vector is less pathogenic. The end result of attenuation is that the risk of toxicity as well as other side-effects is decreased, when the bacterium or bacterial vector is administered to the patient.

Bacteriocin: As used herein, a bacteriocin is a bacterial proteinaceous toxin with selective activity, in that its bacterial host is immune to the toxin. Bacteriocins may be encoded by the bacterial genome or by a plasmid, may be toxic to a broad or narrow range of other bacteria, and may have a simple structure comprising one or two subunits or may have a multi-subunit structure. In addition, a host expressing bacteriocin has immunity against the bacteriocin.

Cytotoxin: As used herein, cytotoxin refers to a compound that results in cell death or cell stasis occurring through apoptosis, necrosis or other mechanism.

Virulence: Virulence is a relative term describing the general ability to cause disease, including the ability to kill normal cells or the ability to elicit septic shock (see specific definition below).

Septic shock: Septic shock is a state of internal organ failure due to a complex cytokine cascade, initiated by TNFα. The relative ability of a bacterium or bacterial vector to elicit TNFα is used as one measure to indicate its relative ability to induce septic shock.

Gene product: Gene product refers to any molecule capable of being encoded by a nucleic acid, including but not limited to, a protein or another nucleic acid, *e.g.*, DNA, RNA dsRNAi, ribozyme, DNazyme, etc. The nucleic acid which encodes for the gene product of interest is not limited to a naturally occurring full-length "gene" having non-coding regulatory elements.

Treatment: In addition to its ordinary meaning, the term treatment encompasses inhibition of progression of symptoms or amelioration of symptoms of a disease or disorder such as a solid tumor cancer.

Tumor-targeted: Tumor-targeted is defined as the ability to distinguish between a cancerous target cell or tissue and the non-cancerous counterpart cell or tissue so that tumor-targeted bacteria, such as *Salmonella* preferentially attache to, infect and/or remain viable in the cancerous target cell or the tumor environment.

Chelating agent sensitivity: Chelating agent sensitivity is defined as the effective concentration at which bacteria proliferation is affected, or the concentration at which the viability of bacteria, as determined by recoverable colony forming units (c.f.u.), is reduced.

Omp-like protein: As used herein, an Omp-like protein includes any bacterial outer membrane protein, or portion thereof (*e.g.*, signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof). In specific embodiments, the Omp-like protein is at least a portion of OmpA, OmpB, OmpC, OmpD, OmpE, OmpF, OmpT, a porin-like protein, PhoA, PhoE, lamB, β-lactamase, an enterotoxin, protein A, endoglucanase, peptidoglycan-associated lipoprotein (PAL), FepA, FhuA, NmpA, NmpB, NmpC, or a major outer membrane lipoprotein (such as LPP), etc.

Release factor: As used herein, a release factor includes any protein, or functional portion thereof which enhances release of bacterial components. In one embodiment a release factor is a bacteriocin release protein. Release factors include but are not limited to Brp, colicin A, colicin D, colicin N, colicin E1-E9, and cloacin DF13.

3.2 OBJECTS OF THE INVENTION

An object of the present invention is to provide methods and compositions for reducing the volume of, or inhibiting the growth of, a sarcoma, carcinoma, or other solid tumor cancer, in a subject by administering to the subject in need of such treatment, an effective dose of irradiation and an effective amount of a live tumor-targeted facultative aerobic or facultative anaerobic bacteria which can replicate at the site of a solid tumor. Other objectives will be appreciated by those skilled in the art and are encompassed by the invention.

The present invention may be understood more fully by reference to the following detailed description, illustrative examples of specific embodiments and the appended figures.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a graph showing the average time, in days, for tumors treated with increasing doses of irradiation (X-rays) to attain 1 gram in weight, with or without concurrent treatment with the *msbB*⁻ attenuated stain YS1646 of *Salmonella*, designated herein as VNP20009. Open circles indicate treatment without *Salmonella*; filled circles indicate treatment with *Salmonella*. The dashed line denotes the anti-tumor effect calculated based on an additive interaction between the radiation and *Salmonella* treatments.

All treatment categories differed significantly from the sham irradiated controls, with p values ranging from <.0001 to .0331. *Salmonella* and X-irradiation treatment differed from X-irradiation treatment alone at each X-ray dose with p values ranging from <.0001 to .0020. See, Section 6.2.2 for details.

FIG. 2 is a graph showing the effects of a single X-ray dose (15 Gy), with or without concurrent treatment with the attenuated *msbB⁻ Salmonella* strain VNP20009, on the growth of mouse melanomas. Data are from the same experiment shown in FIG. 1. Open squares: control mice (no *Salmonella* or X-irradiation treatment); open circles: *Salmonella* treatment only; filled squares: X-irradiation treatment only; filled circles: *Salmonella* treatment and X-irradiation treatment. The numbers shown with the points represent the number of surviving mice/initial number of mice. See, Section 6.2.2 for details.

FIG. 3 is a graph showing the effects of a single X-ray dose (15 Gy), with or without concurrent treatment with the attenuated *msbB⁻ Salmonella* strain VNP20009, on the growth of human colon carcinomas in mice. Open squares: control mice, no treatment; open circles: *Salmonella* treatment only; filled squares: X-irradiation treatment only; filled circles: *Salmonella* treatment and X-irradiation treatment. See, Section 6.2.2 for details.

FIG. 4 is a graph showing the effects of multiple X-ray treatments, to a cumulative dose of 25 Gy, with or without concurrent treatment with the attenuated *Salmonella* strain YS1456 (ATCC Accession No. 202164), on the growth of B16F10 melanoma tumors in mice. Open squares: control mice, no treatment; open circles: *Salmonella* treatment only; filled squares: X-ray treatment only; filled circles: *Salmonella* treatment and X-ray treatment. See, Section 6.2.3 for details.

FIG. 5 is a graph showing the effects of multiple (fractionated) X-ray treatments, to a cumulative dose of 25 Gy, with or without concurrent treatment with the attenuated *Salmonella* strain YS1456, on the growth of EMT-6 mammary carcinoma tumors in mice. Open squares: control mice, no treatment; open circles: *Salmonella* treatment only; filled squares: X-ray treatment only; filled circles: *Salmonella* treatment and X-ray treatment. See, Section 6.2.3 for details.

FIG. 6 is a graph showing the effects of multiple X-ray treatments, to a cumulative dose of 50 Gy with or without concurrent treatment with the attenuated *Salmonella* strain YS1456, on growth of B16F10 melanoma tumors in mice. Open squares: control mice, no treatment; open circles: *Salmonella* treatment only; filled squares: X-ray treatment only; filled circles: *Salmonella* treatment and X-ray treatment. See, Section 6.2.3 for details.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a combination treatment method for reducing the volume of, or inhibiting the growth of, a sarcoma, carcinoma, or other solid tumor cancer, in a subject by administering to the subject in need of such treatment, an effective dose of irradiation and an effective amount of tumor-targeted facultative aerobic or facultative anaerobic bacteria. In one embodiment, the tumor-targeted bacteria are super-infective. In another embodiment, the bacteria have been genetically engineered to express a gene product(s) of interest which gene product aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer. The gene product of interest is selected from the group consisting of proteinaceous and nucleic acid molecules. In a preferred embodiment, the bacteria are attenuated. In another preferred embodiment, the bacteria are tumor-targeted, attenuated bacteria expressing a gene product of interest which aids in reducing the volume, or inhibiting the growth of the tumor.

5.1 USEFUL BACTERIA

The bacteria useful in the present invention are those facultative aerobic and facultative anaerobic bacteria which are able to differentiate between cancerous cells and non-cancerous counterpart cells or distinguish between the environment around a solid tumor from a non-cancerous counterpart environment. For example, the bacteria are able to differentiate between melanoma cells and melanocytes or between colon cancer cells and normal colon epithelial cells. Illustrative examples of bacteria useful in the present invention as tumor-targeted bacteria and/or for isolation of super-infective, tumor-targeted bacteria include, but are not limited to the following facultative aerobes and anaerobes: *Escherichia coli*, including but not limited to pathogenic, including uropathogenic *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis*. While, for ease of explanation, the description below refers specifically to *Salmonella*, the methods of and compositions used in the invention are in no way intended to be restricted to *Salmonella* but rather encompass any and all of the bacteria taught herein as useful.

Factors contributing to attenuation and tumor-targeting are described herein and may be used to construct or select an appropriate bacterial strain for use in the methods of the invention. For example, methods to select and isolate tumor-targeted bacteria are described in section 6.1, and methods to attenuate bacteria are described in section 6.2 of International publication WO96/40238, which is incorporated herein by reference in its entirety. Examples of attenuated tumor-targeted bacteria are also described in International Application WO99/13053, which is incorporated herein by reference in its entirety.

In certain embodiments of the invention, a bacterial strain may be modified by methods known in the art to be attenuated or highly attenuated. In a preferred embodiment of the invention, the attenuated, tumor-targeted bacterial strain which is modified to encode one or more gene products is *Salmonella*.

Salmonella spp. are particularly useful strains for the present invention, since they show natural preference for attachment to and penetration into certain solid tumor cancer cells in tissue culture, as opposed to non-cancerous counterpart cells. (The term "*Salmonella*" is used generically herein to refer to any *Salmonella* species). Since these bacteria, such as *Salmonella*, have a natural ability to distinguish between cancerous cells and their non-cancerous counterpart cells, as well as tumor environment, they are directly applicable to the methods for treatment according to the present invention.

Bacteria such as *Salmonella* is a causative agent of disease in humans and animals. One such disease that can be caused by *Salmonella* is sepsis, which is a serious problem because of the high mortality rate associated with the onset of septic shock (R.C. Bone, 1993, Clinical Microbiol. Revs. 6:57-68). Therefore, to allow the safe use of *Salmonella* in the present invention, the bacteria such as *Salmonella* are attenuated in their virulence for causing disease. In the present application, attenuation, in addition to its traditional definition in which a bacterium is modified so that the bacterium is less pathogenic, is intended to include also the modification of a bacterial strain so that a lower titer of that derived bacterial strain can be administered to a patient and still achieve comparable results as if one had administered a higher titer of the parental bacterial strain. The end result serves to reduce the risk of toxic shock or other side effects due to administration of the strain to the patient. Such attenuated bacteria are isolated by means of a number of techniques. For example, attenuation can be achieved by the deletion or disruption of DNA sequences which encode for virulence factors that insure survival of the bacteria in the host cell, especially macrophages and neutrophils. Such deletion or disruption techniques are well known in the art and include, for example, homologous recombination, chemical mutagenesis, radiation mutagenesis, or transposon mutagenesis. Those virulence factors that are associated with survival in macrophages are usually specifically expressed within the macrophages in response to stress signals, for example, acidification, or in response to host cell defensive mechanisms such macropinocytosis (Fields *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:5189-5193). Table 4 of International Publication WO 96/40238 is an illustrative list of *Salmonella* virulence factors whose deletion results in attenuation.

Yet another method for the attenuation of the bacteria, such as *Salmonella*, is to modify substituents of the bacteria which are responsible for the toxicity of that bacteria.

For example, lipopolysaccharide (LPS) or endotoxin is primarily responsible for the pathological effects of bacterial sepsis. The component of LPS which results in this response is lipid A ("LA"). Elimination or mitigation of the toxic effects of LA results in an attenuated bacteria since 1) the risk of septic shock in the patient is reduced and 2) higher levels of the bacterial strain can be tolerated.

Altering the LA content of bacteria, such as *Salmonella*, can be achieved by the introduction of mutations in the LPS biosynthetic pathway. Several enzymatic steps in LPS biosynthesis and the genetic loci controlling them in *Salmonella* have been identified (Raetz, 1993, J. Bacteriol. 175:5745-5753 and references therein), as well as corresponding mutants. One such illustrative mutant is *firA*, a mutation within the gene that encodes the enzyme UDP-3-O(R-30 hydroxymyristoyl)-glycocyamine N-acyltransferase, which regulates the third step in endotoxin biosynthesis (Kelley *et al.*, 1993, J. Biol. Chem. 268:19866-19874). Bacterial strains bearing this type of mutation produce a lipid A that differs from wild-type lipid A in that it contains a seventh fatty acid, a hexadecanoic acid (Roy and Coleman, 1994, J. Bacteriol. 176:1639-1646). Roy and Coleman demonstrated that in addition to blocking the third step in endotoxin biosynthesis, the *firA*$^-$ mutation also decreases enzymatic activity of lipid A 4' kinase that regulates the sixth step of lipid A biosynthesis.

In addition to being attenuated, the bacteria of the invention are tumor-targeted, *i.e.*, the bacteria preferentially attaches to, infect, and/or remain viable in a tumor or tumor cell versus a normal tissue, non-tumor or non-tumor cell. Suitable methods for obtaining attenuated tumor-targeted bacteria are described in Section 6.1 (pages 25-32; tumor-targeting) and Section 6.2.2 (pages 43-51; attenuation) of International Publication WO 96/40238, which are incorporated by reference herein in its entirety. As the resulting bacteria are highly specific and super-infective, the difference between the number of infecting bacteria found at the target tumor or tumor cell as compared to the non-cancerous counterparts becomes larger and larger as the dilution of the bacterial culture is increased such that lower titers of bacteria can be used with positive results. The techniques described in International Publication WO 96/40238 can also be used to produce attenuated tumor-targeted *Salmonella* or non-*Salmonella* bacterial strains.

An illustrative example of an attenuated tumor-targeted bacterium having an LPS pathway mutant is the *msbB*$^-$ *Salmonella* mutant described in International Publication WO 99/13053, which is incorporated herein by reference in its entirety; see especially Section 6.1.2 which describes the characteristic of the *msbB- Salmonella* mutant. One characteristic of the *msbB*$^-$ *Salmonella* is decreased ability to induce a TNFα response compared to the wild-type bacterial strain. The *msbB*$^-$ *Salmonella* induce TNFα expression

at levels of about 5 percent to about 40 percent compared to the levels induced by wild-type *Salmonella*.

The TNFα response induced by whole bacteria or isolated or purified LPS can be assessed *in vitro* or *in vivo* using commercially available assay systems such as by enzyme linked immunoassay (ELISA). Comparison of TNFα production on a per colony forming unit ("c.f.u.") or on a μg/kg basis, is used to determine relative activity. Lower TNFα levels on a per unit basis indicate decreased induction of TNFα production. In a preferred embodiment, the *msbB⁻ Salmonella* strain is modified to express a gene product of interest which aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer. See, Section 5.1.1, *infra*.

The present invention also encompasses the use of derivatives of *msbB⁻* attenuated tumor-targeted *Salmonella* mutants.

The stability of the attenuated phenotype is important such that the strain does not revert to a more virulent phenotype during the course of treatment of a patient. Such stability can be obtained, for example, by providing that the virulence gene is disrupted by deletion or other non-reverting mutations on the chromosomal level rather than epistatically.

Another method of insuring the attenuated phenotype is to engineer the bacteria such that it is attenuated in more than one manner, *e.g.*, a mutation in the pathway for lipid A production, such as the *msbB⁻* mutation (International Publication WO 99/13053) and one or more mutations to auxotrophy for one or more nutrients or metabolites, such as uracil biosynthesis, purine biosynthesis, and/or arginine biosynthesis as described by Bochner, 1980, J. Bacteriol. 143:926-933. In a preferred embodiment, the tumor-targeted *msbB⁻ Salmonella* strain is also auxotrophic for purine. In certain embodiments, the attenuated tumor-targeted bacteria are attenuated by the presence of a mutation in *aroA, msbB, purI* or *serC*. In another embodiment the attenuated tumor targeted bacteria are attenuated by the presence of a deletion in *aroA, msbB, purI* or *serC*.

In another aspect of this embodiment, the bacteria may be attenuated such that they are more susceptible to an anti-bacterial agent, such as an antibiotic. In this aspect, the bacteria are mutated in a gene that allows the bacteria to pump-out antibiotics, *e.g.*, *acrA*, *acrB*, *acrC*, *acrD*. In other words, when such gene is mutated, the bacteria are more sensitive to antibiotics.

Accordingly, any attenuated, tumor-targeted bacteria may be used in the methods of the invention.

In addition to being attenuated and tumor-targeted, according to one preferred embodiment, the bacteria are genetically modified, *i.e.*, mutated, such that the

bacteria are more responsive to a DNA damaging agent. The mutation may be in a gene involved in DNA repair or recombination, such as *recN*, or may be in a gene involved in the prevention of damage to DNA by destroying oxygen free radicals, such as *oxyR* or *soxR*.

5.1.1 SCREENING FOR DNA DAMAGING AGENT-SENSITIVE BACTERIA

The present invention is also directed to a method for screening to identify bacteria that are more responsive to a DNA damaging agent using the halo assay described by Pugsley and Oudega, 1987, "Methods for Studying Colicins and Their Plasmids", pp. 105-161, In Plasmids, a Practical Approach, K.G. Hardy (ed.), IRL Press, Oxford, UK; and by Gilson et al., 1990, EMBO J. 9:3875-3884. In one embodiment, the method comprises mutating a bacterial culture containing a plasmid coding for the colicin E3 toxin under the control of a SOS-type promoter to produce a mutated bacterial culture, plating an individual colony clone of the mutated bacterial culture on to a bacterial culture plate containing a lawn of E3 toxin sensitive bacteria, culture for a time sufficient for background expression of the E3 toxin, measuring a background clearing zone (halo formation) around the colony, exposing the colony to a DNA damaging agent to induce expression of the E3 toxin, culturing the bacterial culture for a time sufficient for induced expression of the E3 toxin, and measuring the induced clearing zone (halo formation) around the colony. Those bacterial cultures having larger clearing zones are more responsive to the DNA damaging agent. In certain aspects of this embodiment, approximately 300 colony clones are plated. In yet other aspects, image analysis is used to measure halo formation. In another aspect of this embodiment, the bacterial culture is not mutated prior to plating the individual colony clone.

In an alternative embodiment, the method comprises mutating a bacterial culture containing a plasmid coding for the colicin E3 toxin under the control of a SOS-type promoter to produce a mutated bacterial culture, plating an individual colony clone of the mutated bacterial culture on to a bacterial culture plate, culturing for a time sufficient for colony formation, transferring a colony to liquid culture, exposing the liquid culture to a DNA damaging agent, culturing for a time sufficient for induced expression of the E3 toxin, diluting the liquid culture, plating the diluted culture on a bacterial culture plate containing a lawn of E3 toxin sensitive bacteria, culturing for a time sufficient for the E3 toxin to detectably kill the E3 sensitive bacteria, and measuring a clearing zone (halo) around the plated bacterial culture. Those bacterial cultures having larger clearing zones are more responsive to the DNA damaging agent. In one aspect of this embodiment, the liquid bacterial culture is centrifuged and the bacteria-free supernatant is plated on the bacterial culture plate. In another aspect of this embodiment, the culture is diluted by at least a factor

of 100. In another aspect of this embodiment, the bacterial culture is not mutated prior to plating the individual colony clone. In one illustrative example, liquid growth medium in microtiter plates (*e.g.*, 48 or 96 well plates) is innoculated with an independent clone of a bacterial culture expressing colicin E3 under the control of a SOS-type promoter, *e.g.*, *recN*, the innoculated culture are exposed to a DNA damaging agent, such as mitomycin, X-irradiation, and the culture is allowed to grow for a time sufficient for expression of the E3 toxin to be induced. The growth culture medium is then diluted at least 100 fold to dilute the secreted E3 toxin and the diluted medium is spotted onto an agar plate containing a lawn of E3 sensitive bacteria. The plate is incubated for a sufficient time for the E3 toxin to detectably kill the sensitive bacteria and the clearing zone produced is measured. The larger the clearing zone, the more E3 that was produced by the strain, which indicates that the strain is more responsive to the DNA damaging agent.

5.1.2 BACTERIA EXPRESSING A GENE PRODUCT

In certain embodiments of the present invention, the methods employ a tumor-targeted bacterial strain described in Section 5.1 that has been genetically modified to express a gene product product(s) of interest which aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer, and that allows for delivery of the gene product of interest to the tumor site. The gene product of interest is selected from the group consisting of proteinaceous and nucleic acid molecules. In various embodiments, the proteinaceous molecule is a cellular toxin (cytotoxic agent), *e.g.*, saporin, cytotoxic necrotic factor-1, cytotoxic necrotic factor-2, a ribosome inactivating protein, or a porin protein, such as gonococcal PI porin protein. In other embodiments, the proteinaceous molecule is an anti-angiogenesis protein, an antibody or an antigen. In yet other embodiments, the proteinaceous molecule is a cytokine, *e.g.*, IL-2, or an anti-angiogenic factor, *e.g.*, endostatin, or a pro-drug converting enzyme, *e.g.*, Herpes Simplex Virus ("HSV") thymidine kinase or cytosine deaminase. The nucleic acid molecule can be double stranded or single stranded DNA or double stranded or single stranded RNA, or a triplex nucleic acid molecule. The nucleic acid molecule can function can function as a ribozyme, DNazyme or antisense nucleic acid, etc.

The nucleic acid encoding a gene product of interest is provided in an expression vector in operative linkage with a selected promoter, and optionally in operative linkage with other elements that participate in transcription, translation, localization, stability and the like.

The nucleic acid molecule encoding the gene product of interest is from about 6 base pairs to about 100,000 base pairs in length. Preferably the nucleic acid

molecule is from about 20 base pairs to about 50,000 base pairs in length. More preferably the nucleic acid molecule is from about 20 base pairs to about 10,000 base pairs in length. Even more preferably, it is a nucleic acid molecule from about 20 pairs to about 4,000 base pairs in length.

The promoter that controls the expression of the gene product should be active or activatible in at the tumor site. The tumor can be in, but is not limited to, a mammalian or avian cell. The mammalian cell can be, but is not limited to, human, canine, feline, equine, bovine, porcine, rodent, etc. The choice of promoter will depend on the type of target cell and the degree or type of expression control desired. Promoters that are suitable for use in the present invention include, but are not limited to, constitutive, inducible, tissue-specific, cell type-specific and temporal-specific and need not necessarily function in a mammalian cell. Another type of promoter useful in the present invention is an event-specific promoter which is active or up-regulated in response to the occurrence of an event, such as viral infection. For example, the HIV LTR is an event specific promoter. The promoter is inactive unless the *tat* gene product is present, which occurs upon HIV infection.

A preferred promoter for use in the present invention is a promoter that is activatable by a DNA damaging agent, *e.g.*, X-ray irradiation. Illustrative examples of such a X-irradiation inducible promoter include, but are not limited to, *recA*, *sulA*, *umuC*, *dinA*, *dinB*, *dinD*, *lexA*, *ruv*, *uvrA*, *uvrB*, *uvrD*, *umuD*, *cea*, *caa*, *recN*, *oxyR*, *soxR*, etc. See, *e.g.*, Schnarr et al., 1991, Biochimie 73: 423-431 for a general review of SOS-responsive promoters. These promoters are in the SOS class (SOS-type) of promoters, which activate transcription in response to a DNA damaging agent.

Other exemplary promoters useful in the present invention include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the cytomegalovirus ("CMV") promoter, the regulatory sequences of the tyrosinase gene which is active in melanoma cells (Siders *et al.*, 1998, Gen. Ther. 5:281-291), the regulatory sequences of the metallothionein gene (Brinster *et al.*, 1982, Nature 296:39-42); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella *et al.*, Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, *et al.*, 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella *et al.*, 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK

(phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), prostate specific antigen gene control region which is active in prostate cells, and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

Another exemplary promoter is one that has enhanced activity in the tumor environment; for example, a promoter that is activated by the anaerobic environment of the tumor such as the P1 promoter of the *pepT* gene. Activation of the P1 promoter is dependent on the FNR transcriptional activator (Strauch *et al.*, 1985, J. Bacteriol. 156:743-751). In a specific embodiment, the P1 promoter is a mutant promoter that is induced at higher levels under anaerobic conditions than the native P1 promoter, such as the pepT200 promoter whose activity in response to anaerobic conditions is induced by CRP-cAMP instead of FNR (Lombardo *et al.*, 1997, J. Bacteriol. 179:1909-1917). In another embodiment, an anaerobically-induced promoter is used, *e.g.*, the *potABCD* promoter. *potABCD* is an operon that is divergently expressed from *pepT* under anaerobic conditions. The promoter in the *pepT* gene responsible for this expression has been isolated (Lombardo *et al.*, 1997, J. Bacteriol. 179:1909-1917) and can be used according to the methods of the present invention.

Yet another exemplary promoter is an antibiotic-induced promoter, such as the *tet* promoter of the Tn10 transposon. In a preferred embodiment, the *tet* promoter is

multimerized, for example, three-fold. Promoter activity would then be induced by administering to a subject who has been treated with the attenuated tumor-targeted bacteria of the invention an appropriate dose of tetracycline. Although the *tet* inducible expression system was initially described for eukaryotic systems such as *Schizosaccharomyces pombe* (Faryar and Gatz, 1992, Current Genetics 21:345-349) and mammalian cells (Lang and Feingold, 1996, Gene 168:169-171), recent studies extend its applicability to bacterial cells. For example, Stieger *et al.*, 1999, Gene 226:243-252) have shown 80-fold induction of the firefly luciferase gene upon tet induction when operably linked to the *tet* promoter. An advantage of this promoter is that it is induced at very low levels of tetracycline, approximately 1/10th of the dosage required for antibiotic activity.

In addition to the promoter, repressor sequences, negative regulators, or tissue-specific silencers can be inserted to reduce non-specific expression of the gene product. Moreover, multiple repressor elements may be inserted in the promoter region. One type of repressor sequence is an insulator sequence. Illustrative examples of repressor sequences which silence background transcription are found in Dunaway *et al.*, 1997, Mol. Cell Biol. 17:182-129; Gdula *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93:9378-9383; Chan *et al.*, 1996, J. Virol. 70:5312-5328. In certain embodiments, sequences which increase the expression of the gene product can be inserted in the expression vector, *e.g.*, ribosome binding sites. Expression levels of the transcript or translated product can be assayed by any method known in the art to ascertain which promoter/repressor sequences affect expression.

In certain embodiments, the gene product of interest is cytotoxic or cytostatic to a cell by inhibiting cell growth through interference with protein synthesis or through disruption of the cell cycle. Such a product may act, for example, by cleaving rRNA or ribonucleoprotein, inhibiting an elongation factor, cleaving mRNA, or other mechanism that reduced protein synthesis to a level such that the cell cannot survive.

Examples of suitable gene products include, without limitation, saporin, the ricins, abrin, other ribosome inactiviting proteins (RIPs), *Pseudomonas* exotoxin, inhibitors of DNA, RNA or protein synthesis, antisense nucleic acids, other metabolic inhibitors (*e.g.*, DNA or RNA cleaving molecules such as DNase and ribonuclease, protease, lipase, phospholipase), prodrug converting enzymes (*e.g.*, thymidine kinase from HSV and bacterial cytosine deaminase), light-activated porphyrin, ricin, ricin A chain, maize RIP, gelonin, *E. coli* cytotoxic necrotic factor-1, *Vibrio fischeri* cytotoxic necrotic factor-1, cytotoxic necrotic factor-2, *Pasteurella multicida* toxin (PMT), cytolethal distending toxin, hemolysin, verotoxin, diphtheria toxin, diphtheria toxin A chain, trichosanthin, tritin, pokeweed antiviral protein (PAP), mirabilis antiviral protein (MAP), Dianthins 32 and 30,

abrin, monodrin, bryodin, shiga, a catalytic inhibitor of protein biosynthesis from cucumber seeds (see, *e.g.*, International Publication WO 93/24620), *Pseudomonas* exotoxin, *E. coli* heat-labile toxin, *E. coli* heat-stable toxin, EaggEC stable toxin-1 (EAST), biologically active fragments of cytotoxins and others known to those of skill in the art. See, *e.g.*, O'Brian and Holmes, Protein Toxins of *Escherichia coli* and *Salmonella* in Escherichia and Salmonella, Cellular and Molecular Biology, Neidhardt et al. (eds.), pp. 2788-2802, ASM Press, Washington, D.C. for a review of *E. coli* and *Salmonella* toxins. Yet other exemplary gene products of interest include, but are not limited to, methionase, aspariginase and glycosidase.

In a mode of this embodiment, the gene product of interest is a pro-drug converting enzyme or nucleic acid encoding the same, *i.e.* an enzyme that modulates the chemical nature of a drug to produce a cytotoxic agent. Illustrative examples of pro-drug converting enzymes are listed on page 33 and in Table 2 of WO 96/40238 by Pawelek *et al.*, which is incorporated herein in its entirety. WO 96/40238 also teaches methods for production of secreted fusion proteins comprising such pro-drug converting enzymes. According the present invention, a pro-drug converting enzyme need not be a secreted protein if co-expressed with a release factor such as BRP (see *infra*). In a specific embodiment, the pro-drug converting enzyme is cytochrome p450 NADPH oxidoreductase which acts upon mitomycin C and porfiromycin (Murray *et al.*, 1994, J. Pharmacol. Exp. Therapeut. 270:645-649). In another mode of the embodiment, the gene product(s) of interest is co-expressed with a release factor such as BRP, and cause the release of co-factors (*e.g.*, NADH, NADPH, ATP, *etc.*) which enhance pro-drug converting enzyme activity. In another mode of the embodiment, a gene product of interest is co-expressed with a release factor such as BRP, leading to the release of an activated drug (*e.g.*, a drug which is activated within the bacterial cytoplasm or periplasm, and then released from the bacterial vector).

Nucleic acid molecules that encode an enzyme that results in cell death or renders a cell susceptible to cell death upon the addition of another product are preferred. Ribosome-inactivating proteins (RIPs), which include ricin, abrin, and saporin, are plant proteins that catalytically inactivate eukaryotic ribosomes. Ribosome-inactivating proteins inactivate ribosomes by interfering with the protein elongation step of protein synthesis. For example, the ribosome-inactivating protein saporin is an enzyme that cleaves rRNA and inhibits protein synthesis. Other enzymes that inhibit protein synthesis are especially well suited for use in the present invention. Any of these proteins, if not derived from mammalian sources, may use mammalian-preferred codons. Preferred codon usage is

exemplified in *Current Protocols in Molecular Biology, infra,* and Zhang et al., 1991, Gene 105: 61.

A nucleic acid molecule encoding a pro-drug converting enzyme may alternatively be used according to the present invention. Pro-drugs are inactive in the host cell until either a substrate or an activating molecule is provided. As used herein, a "pro-drug converting enzyme" is a compound that metabolizes or otherwise converts an inactive, nontoxic compound to a biologically, pharmaceutically, therapeutically, of toxic active form of the compound or is modified upon administration to yield an active compound through metabolic or other processes. Most typically, a pro-drug converting enzyme activates a compound with little or no cytotoxicity into a toxic compound. Two of the more often used pro-drug converting molecules, both of which are suitable for use in the present invention, are HSV thymidine kinase and *E. coli* cytosine deaminase.

Briefly, a wide variety of gene products which either directly or indirectly activate a compound with little or no cytotoxicity into a toxic product may be utilized. Representative examples of such gene products include HSVTK (herpes simplex virus thymidine kinase) and VZVTK (varicella zoster virus thymidine kinase), which selectively phosphorylate certain purine arabinosides and substituted pyrimidine compounds. Phosphorylation converts these compounds to metabolites that are cytotoxic or cytostatic. For example, exposure of the drug ganciclovir, acyclovir, or any of their analogues (*e.g.*, FIAU, FIAC, DHPG) to cells expressing HSVTK allows conversion of the drug into its corresponding active nucleotide triphosphate form.

Other gene products may be utilized in accordance with the present invention include *E. coli* guanine phosphoribosyl transferase, which converts thioxanthine into toxic thioxanthine monophosphate (Besnard et al., *Mol. Cell. Biol. 7*: 4139-4141, 1987); alkaline phosphatase, which converts inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (*e.g., Fusarium oxysporum*) or bacterial cytosine deaminase, which converts 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS, 89*:33, 1992); carboxypeptidase G2, which cleaves glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which converts phenoxyacetabide derivatives of doxorubicin and melphalan to toxic compounds (see generally, Vrudhula et al., 1993, J. of Med. Chem. 36(7):919-923; Kern et al., 1990, Canc. Immun. Immunother. 31(4):202-206). Moreover, a wide variety of *Herpesviridae* thymidine kinases, including both primate and non-primate herpesviruses, are suitable. Such herpesviruses include Herpes Simplex Virus Type 1 (McKnight et al., 1980, Nuc. Acids Res. 8:5949-5946), Herpes Simplex Virus Type 2 (Swain and Galloway, 1983, J.

Virol. 46:1045-1050), Varicella Zoster Virus (Davison and Scott, 1986, J. Gen. Virol. 67:1759-1816), marmoset herpesvirus (Otsuka and Kit, 1984, Virology 135:316-330), feline herpesvirus type 1 (Nunberg et al., 1989, J. Virol. 63:3240-3249), pseudorabies virus (Kit and Kit, 1985, U.S. Patent No, 4,514,497), equine herpesvirus type 1 (Robertson and Whalley, 1988, Nuc. Acids Res. 16:11303-11317), bovine herpesvirus type 1 (Mittal and Field, 1989, J. Virol. 70:2901-2918) turkey herpesvirus (Martin et al., 1989, J. Virol. 63:2847-2852), Marek's disease virus (Scott et al., 1989, J. Gen. Virol. *70*:3055-3065), herpesvirus saimiri (Honess et al., 1984, J. Gen. Virol. 70:207-311). Such herpesviruses may be readily obtained from commercial sources such as the American Type culture collection ("ATCC", Manassas, VA).

Furthermore, as indicated above, a wide variety of inactive precursors may be converted into active inhibitors. For example, thymidine kinase can phosphorylate nucleosides (*e.g.* dT) and nucleoside analogues such as ganciclovir (9-{[2-hydroxy-1-(hydroxymethyl)ethoxyl methyl} guanosine), famciclovir, buciclovir, penciclovir, valciclovir, acyclovir (9-[2-hydroxy ethoxy)methyl] guanosine), trifluorothymidine, 1-[2-deoxy, 2-fluor, beta-D-arabino furanosyl]-5-iodouracil, ara-A (adenosine arabinoside, vivarabine), 1-beta-D-arabinofuranoxyl thymine, 5-ethyl-2'-deoxyuridine), AZT (3' azido-3' thymidine), ddC (dideoxycytidine), AIU (5-iodo-5' amino 2', 5'-dideoxyuridine) and AraC (cytidine arabinoside). Other gene products may render a cell susceptible to toxic agents. Such products include viral proteins, and channel proteins that transport drugs.

Moreover, a cytocide-encoding agent may be constructed as a pro-drug, which when expressed in the proper cell type is processed or modified to an active form. For example, the saporin gene may be constructed with an N- or C-terminal extension containing a protease-sensitive site. The extension renders the initially translated protein inactive and subsequent cleavage in a cell expressing the appropriate protease restores enzymatic activity.

The nucleotide sequences of the genes encoding these gene products are well known (*see* GenBank). A nucleic acid molecule encoding one of the gene products may be isolated by standard methods, such as amplification (*e.g.*, PCR), probe hybridization of genomic or cDNA libraries, antibody screenings of expression libraries, chemically synthesized or obtained from commercial or other sources.

In another mode of the embodiment, the gene product of interest is cytotoxic or cytostatic to a cell by inhibiting the production or activity of a protein involved in cell proliferation, such as an oncogene or growth factor, (*e.g.*, bFGF, int-2, hst-1/K-FGF, FGF-5, hst-2/FGF-6, FGF-8) or cellular receptor or ligand. The inhibition can be at the level of transcription or translation (mediated by another gene product that is a ribozyme or triplex

DNA), or at the level of protein activity (mediated by another gene product that is an inhibitor of a growth factor pathway, such as a dominant negative mutant).

Nucleic acid molecules and oligonucleotides for use as described herein can be synthesized by any method known to those of skill in this art (see, *e.g.*, International Publication WO 93/01286, U.S. Patent Nos. 5,218,088; 5,175,269; 5,109,124). Identification of oligonucleotides and ribozymes for use as antisense agents and DNA encoding genes for targeted delivery for genetic therapy involve methods well known in the art. For example, the desirable properties, lengths and other characteristics of such oligonucleotides are well known. Antisense oligonucleotides may be designed to resist degradation by endogenous nucleolytic enzymes using linkages such as phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and the like (*see, e.g.*, Stein in: *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression,* Cohen, Ed, Macmillan Press, London, pp. 97-117, 1989); Jager et al., 1988, Biochemistry 27:7237).

Antisense nucleotides are oligonucleotides that bind in a sequence-specific manner to nucleic acids, such as mRNA or DNA. When bound to mRNA that has complementary sequences, antisense prevents translation of the mRNA (see, *e.g.*, U.S. Patent Nos. 5,168,053; 5,190,931; 5,135,917; 5,087,617). Triplex molecules refer to single DNA strands that bind duplex DNA forming a colinear triplex molecule, thereby preventing transcription (see, *e.g.*, U.S. Patent No. 5,176,996).

Particularly useful antisense nucleotides and triplex molecules are molecules that are complementary to bind to the sense strand of DNA or mRNA that encodes a protein involved in cell proliferation, such as an oncogene or growth factor, (*e.g.*, bFGF, int-2, hst-1/K-FGF, FGF-5, hst-2/FGF-6, FGF-8). Other useful antisense oligonucleotides include those that are specific for IL-8 (see, *e.g.*, U.S. Patent No. 5,241,049), c-*src*, c-*fos* H-*ras* (lung cancer), K-*ras* (breast cancer), urokinase (melanoma), BCL2 (T-cell lymphoma), IGF-1 (glioblastoma), IGF-1 (glioblastoma), IGF-1 receptor (glioblastoma), TGF-β1, and CRIPTO EGF receptor (colon cancer). These particular antisense plasmids reduce tumorigenicity in athymic and syngeneic mice.

A ribozyme is an RNA molecule that specifically cleaves RNA substrates, such as mRNA, resulting in inhibition or interference with cell growth or expression. There are at least five known classes of ribozymes involved in the cleavage and/or ligation of RNA chains. Ribozymes can be targeted to any RNA transcript and can catalytically cleave that transcript (see, *e.g.*, U.S. Patent No. 5,272,262; U.S. Patent No. 5,144,019; and U.S. Patent Nos. 5,168,053, 5,180,818, 5,116,742 and 5,093,246).

In another embodiment, the nucleic acid molecule encodes for a tumor-associated antigen. An example of a tumor-associated antigen is a molecule specifically expressed by a tumor cell and is not expressed in the non-cancerous counterpart cell or is expressed in the tumor cell at a higher level than in the non-cancerous counterpart cell. Illustrative examples of tumor associated antigens are described in Kuby, *Immunology*, W.H. Freeman and Company, New York, NY, pp. 515-520 and Robbins and Kawakami, 1996, Curr. Opin. Immunol. 8:628-363, which are incorporated by reference herein, and include melanocyte lineage proteins such as gp100, MART-1/MelanA, TRP-1 (gp75), tyrosinase; tumor-specific, widely shared antigens such as MAGE-1, MAGE-3, BAGE, GAGE-1, -2, N-acetylglucosaminyltransferase-V, p15; tumor-specific, mutated antigens such as β-catenin, MUM-1, CDK4; and non-melanoma antigens such as HER-2/neu (breast and ovarian carcinoma), human papilloma virus-E6, E7 (cervical carcinoma), MUC-1 (breast, ovarian and pancreatic carcinoma). Other examples of tumor associated antigens are known to those of skill in the art.

In yet another embodiment, the gene product of interest is an antibody molecule that is preferably expressed within the target cell; hence, these antibody molecules have been given the name "intrabodies." Conventional methods of antibody preparation and sequencing are useful in the preparation of intrabodies and the nucleic acid sequences encoding same; it is the site of action of intrabodies that confers particular novelty on such molecules. (For a review of various methods and compositions useful in the modulation of protein function in cells via the use of intrabodies, see International Application WO 96/07321).

Intrabodies are antibodies and antibody derivatives (including single-chain antibodies or "SCA") introduced into cells as transgenes that bind to and incapacitate an intracellular protein in the cell that expresses the antibodies. As used herein, intrabodies encompass monoclonals, single chain antibodies, V regions, and the like, as long as they bind to the target protein. Intrabodies to proteins involved in cell replication, tumorigenesis, and the like (*e.g.*, HER2/neu, VEGF, VEGF receptor, FGF receptor, FGF) are especially useful. The intrabody can also be a bispecific intrabody. Such a bispecific intrabody is engineered to recognize both (1) the desired epitope and (2) one of a variety of "trigger" molecules, *e.g.*, Fc receptors on myeloid cells, and CD3 and CD2 on T cells, that have been identified as being able to cause a cytotoxic T cell to destroy a particular target.

For example, antibodies to HER2/neu (also called erbB-2) may be used to inhibit the function of this protein. HER2/neu has a pivotal role in the progression of certain tumors, human breast, ovarian and non-small lung carcinoma. Thus, inhibiting the

function of HER2/neu may result in slowing or halting tumor growth (see, *e.g.* U.S. Patent No. 5,587,458).

In another mode of the embodiment, a gene product of interest is an inhibitor of inducible nitric oxide synthase (NOS) or of endothelial nitic oxide synthase. Nitric oxide (NO) is implicated to be involved in the regulation of vascular growth and in arterosclerosis. NO is formed from L-arginine by nitric oxide synthase (NOS) and modulates immune, inflammatory and cardiovascular responses.

In certain other embodiments, the gene product of interest can be an anti-angiogenic factor, such as endostatin. Additional exemplary anti-angiogenic factors include, angiostatin, apomigren, anti-angiogenic antithrombin III, the 29 kDa N–terminal and a 40 kDa and/or 29 kDa C-terminal proteolytic fragments of fibronectin, a uPA receptor antagonist, the16 kDa proteolytic fragment of prolactin, the 7.8 kDa proteolytic fragment of platelet factor-4, the anti-angiogenic 13 amino acid fragment of platelet factor-4, the anti-angiogenic 14 amino acid fragment of collagen I, the anti-angiogenic 19 amino acid peptide fragment of Thrombospondin I, the anti-angiogenic 20 amino acid peptide fragment of SPARC, RGD and NGR containing peptides, the small anti-angiogenic peptides of laminin, fibronectin, procollagen and EGF, and peptide antagonists of integrin $\alpha_v\beta_3$ and the VEGF receptor. The anti-angiogenic factor can also be a Flt-3 ligand or nucleic acid encoding the same.

Cytokine immunotherapy is a modification of immunogene therapy and involves the administration of tumor cell vaccines that are genetically modified *ex vivo* or *in vivo* to express various cytokine genes. In animal tumor models, cytokine gene transfer resulted in significant antitumor immune response (Fearon, et al., 1990, Cell 60:387-403; Wantanabe, et al., 1989, Proc. Nat. Acad. Sci. USA, 86:9456-9460). Thus, in the present invention, the gene product of interest is a cytokine, such as IL-1, IL-2, IL-4, IL-5, IL-15, IL-18, IL-12, IL-10, GM-CSF, INF-γ, INF-α, SLC, endothelial monocyte activating protein-2 (EMAP2), MIP-3α, MIP-3β, or an MHC gene, such as HLA-B7. Addtionally, other exemplary cytokines include members of the TNF family, including but not limited to tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), TNF-α-related apoptosis-inducing ligand (TRAIL), TNF-α-related activation-induced cytokine (TRANCE), TNF-α-related weak inducer of apoptosis (TWEAK), CD40 ligand (CD40L), LT-α, LT-β, OX4OL, CD4OL, FasL, CD27L, CD30L, 4-1BBL, APRIL, LIGHT, TL1, TNFSF16, TNFSF17, and AITR-L, or a functional portion thereof. See, *e.g.*, Kwon et al., 1999, Curr. Opin. Immunol. 11:340-345 for a general review of the TNF family. Delivery of these gene products will modulate the immune system, increasing the potential for host antitumor immunity. Alternatively, nucleic acid molecules encoding costimulatory

molecules, such as B7.1 and B7.2, ligands for CD28 and CTLA-4 respectively, can also be delivered to enhance T cell mediated immunity. These gene products can be co-delivered with cytokines, using the same or different promoters and optionally with an internal ribosome binding site. Similarly, α-1,3-galactosyl transferase expression on tumor cells allows complement-mediated cell killing.

In a preferred mode of this embodiment, the gene product of interest is a bacteriocin (*see, e.g.*, Konisky, 1982, Ann. Rev. Microbiol. 36:125-144) which acts as a cytotoxin when delivered to a mamalian cell. In a preferred mode of this embodiment of the invention, the bacteriocin is a colicin, most preferably colicin E3 or V, although colicins A, E1, E2, Ia, Ib, K, L, M (see, Konisky, 1982, Ann. Rev. Microbiol. 36:125-144) can alternatively be used. In another preferred mode of this embodiment, the bacteriocin is a cloacin, most preferably cloacin DF13. Other bacteriocins that are encompassed for use in the invention include, but are not limited to, pesticin A1122, staphylococcin 1580, butyricin 7423, vibriocin (see, *e.g.*, Jayawardene and Farkas-Himsley, 1970, J. Bacteriology 102:382-388), pyocin R1 or AP41, and megacin A-216. In a highly preferred embodiment, the bacteriocin is BRP (Bacteriocin Release Protein) from *Enterococus cloacae.*

For example, colicin E3 (ColE3) has been shown to have a profoundly cytotoxic effect on mammalian cells (see, Smarda *et al.*, 1978, Folia Microbiol. 23:272-277), including a leukemia cell model system (see, Fiska *et al.*, 1978, Experimentia 35: 406-407). ColE3 cytotoxicity is a function of protein synthesis arrest, mediated by inhibition of 80S ribosomes (Turnowsky *et al.*, 1973, Biochem. Biophys. Res. Comm. 52:327-334). More specifically, ColE3 has ribonuclease activity (Saunders, 1978, Nature 274:113-114). In its naturally occurring form, ColE3 is a 60kDa protein complex consisting of a 50kDa and a 10kDa protein in a 1:1 ratio, the larger subunit having the nuclease activity and the smaller subunit having inhibitory function of the 50kDa subunit. Thus, the 50kDa protein acts as a cytotoxic protein (or toxin), and the 10kDa protein acts as an anti-toxin. Accordingly, in one embodiment, when ColE3 is used as a gene product of interest, the larger ColE3 subunit or an active fragment thereof is expressed alone or at higher levels than the smaller subunit. In a preferred mode of the embodiment, ColE3 expression is accompanied by BRP expression to enhance release into the tumor environment (see *infra*). In yet another embodiment of the invention, the ColE3 50kDa toxin and 10kDa anti-toxin are encoded on a single plasmid within an attenuated tumor-targeted bacteria, such as *Salmonella*. In this embodiment, the toxin/anti-toxin can act as a selection system for the *Salmonella* which carry the plasmid, such that *Salmonella* which lose the plasmid are killed by the toxin. In another embodiment, the 10kDa anti-toxin is on the chromosome, separate

from the colE3 toxin on the plasmid, resulting in a barrier to transmission of other bacteria (see, Diaz *et al.*, 1994, Mol. Microbiol. 13:855-861).

In another preferred mode of this embodiment, the bacteriocin is cloacin DF13. Cloacin DF13 functions in an analogous manner to ColE3. The protein complex is of 67kDa molecular weight. The individual components are 57kDa and 9kDa in size. In addition to its ribonuclease activity, DF13 can cause the leakage of cellular potassium.

In yet another preferred mode of this embodiment, the bacteriocin is colicin V (see, e.g., Pugsley, A.P. and Oudega, B. "Methods for Studying Colicins and their Plasmids" Plasmids a Practical Approach, 1987, ed. by K.G. Hardy; Gilson, L. *et al.*, 1990, EMBO J. 9:3875-3884).

In another embodiment, the bacteriocin is selected from the group consisting of colicin E2 (a dual subunit colicin similar to ColE3 in structure but with endonuclease rather than ribonuclease activity); colicins A, E1, Ia, Ib, or K, which form ion-permeable channels, causing a collapse of the proton motive force of the cell and leading to cell death; colicin L which inhibits protein, DNA and RNA synthesis; colicin M which causes cell sepsis by altering the osmotic environment of the cell; pesticin A1122 which functions in a manner similar to colicin B function; staphycoccin 1580, a pore-forming bacteriocin; butyricin 7423 which indirectly inhibits RNA, DNA and protein synthesis through an unknown target; Pyocin P1, or protein resembling a bacteriophage tail protein that kills cells by uncoupling respiration from solute transport; Pyocin AP41 which has a colicin E2-like mode of action; and megacin A-216 which is a phospholipase that causes leakage of intracellular material (for a general review of bacteriocins, see Konisky, 1982, Ann. Rev. Microbiol. 36:125-144).

Accordingly, a gene product of interest may comprise any bacteriocin described herein or known in the art.

In yet another mode of this embodiment, the bacteriocin is BRP. The cytotoxic activity of BRP is mediated by the release of cellular components; therefore, its use is discussed *infra*.

In a particular embodiment, the gene product of interest comprises a number of viral gene products. For example, the gene product of interest comprises all the viral proteins encoded by an adenovirus, reovirus or herpesvirus genome. In a particular example, the gene product of interest is all the viral proteins encoded by an adenovirus genome except for the E1B viral protein such that this particular adenovirus can only replicate in a mammalian cell lacking p53 activity.

In certain embodiments, the gene product of interest is a fragment, analog, or variant of the wild-type full-length gene product, or a nucleic acid encoding the same. The

derivative, analog or variant is functionally active, *e.g.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type gene product. As one example, such derivatives, analogs or variants which have the desired therapeutic properties can be used to inhibit tumor growth. Derivatives or analogs of a gene product of interest can be tested for the desired activity by procedures known in the art, including those described herein.

In particular, variants can be made by altering gene sequences by substitutions, additions (*e.g.*, insertions) or deletions that provide molecules having the same or increased anti-tumor function relative to the wild-type gene product. For example, the variants of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of the gene product, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change, *i.e.*, the altered sequence has at least one conservative substitution.

Any of the gene product of interest-encoding nucleic acids that are of mammalian origin can be altered to employ bacterial codon usage by methods known in the art. Preferred codon usage is exemplified in Current Protocols in Molecular Biology, Green Publishing Associates, Inc., and John Wiley & Sons, Inc. New York, and Zhang *et al.*, 1991, Gene 105: 61.

In certain embodiments, the gene product of interest is expressed as a fusion protein. In a specific embodiment, a gene product is constructed as a chimeric or fusion protein comprising the gene product or a fragment thereof joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one mode of this embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid comprising or encoding the gene product of interest, *e.g.*, comprising a TNF encoding sequence, joined in-frame to a coding sequence for a different protein. Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product into the expression vehicle of choice by methods commonly known in the art. Chimeric nucleic acids comprising portions of a nucleic acid comprising or encoding a gene product of interest fused to any heterologous protein-encoding sequence may be constructed. In a specific mode, the fusion protein comprises an affinity tag such as a hexahistidine tag, or other affinity tag that may be used in purification, isolation, identification, or assay of expression. In another specific mode of this embodiment, the fusion protein comprises a protease cleavage site such as a metal protease or serine cleavage site. In this particular mode, it is in

some cases preferred that a protease site corresponding to a protease which is active at the site of a tumor is constructed into a fusion protein of the invention. In certain embodiments, an gene product of interest is constructed as a fusion protein to an Omp-like protein, or portion thereof (*e.g.*, signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof; see *infra*, Section 3.1 for definition of "Omp-like protein").

In a preferred embodiment, a gene product of interest is expressed as a fusion protein with an outer membrane protein (Omp-like protein). Bacterial outer membrane proteins are integral membrane proteins of the bacterial outer membrane, possess multiple membrane-spanning domains and are often attached to one or more lipid moieties. Outer membrane proteins are initially expressed in precursor form (the pro-Omp) with an amino terminal signal peptide that directs the protein to the membrane, upon which the signal peptide is cleaved by a signal peptidase to produce the mature protein. In one embodiment, a gene product of interest is constructed as a fusion protein with an Omp-like protein. In this embodiment, the gene product has enhanced delivery to the outer membrane of the bacteria. Without intending to be limiting as to mechanism, the Omp-like protein is believed by the inventors to act as an anchor or tether for the gene product of interest to the outer membrane, or serves to localize the protein to the bacterial outer membrane. In one embodiment, the fusion of a gene product of interest to an Omp-like protein is used to enhance localization of an effector molecule to the periplasm. In another embodiment, the fusion of a gene product of interest to an Omp-like proteins is used to enhance release of the gene product. In several embodiments, the gene product of interest is constructed as a fusion protein to an Omp-like protein, or portion thereof (*e.g.*, signal sequence, leader sequence, periplasmic region, transmembrane domain, multiple transmembrane domains, or combinations thereof). In specific embodiments, the Omp-like protein is at least a portion of OmpA, OmpB, OmpC, OmpD, OmpE, OmpF, OmpT, a porin-like protein, PhoA, PhoE, lamB, β-lactamase, an enterotoxin, protein A, endoglucanase, peptidoglycan-associated lipoprotein (PAL), FepA, FhuA, NmpA, NmpB, NmpC, or a major outer membrane lipoprotein (such as LPP), etc. In certain embodiments of the invention, the signal sequence is constructed to be more hydrophobic (*e.g.*, by the insertion or replacement of amino acids within the signal sequence to hydrophobic amino acids, *e.g.*, Leucine).

In other embodiments of the invention, a fusion protein of the invention comprises a proteolytic cleavage site. The protolytic cleavage site may be endogenous to the gene product or endogenous to the Omp-like protein, or the proteolytic cleavage site may be constructed into the fusion protein. In certain specific embodiments, the Omp-like

protein of the invention is a hybrid Omp comprising structural elements that originate from separate proteins.

In an exemplary mode of the embodiment, the Omp-like protein is OmpA; the same principles used in the construction of OmpA-like fusion proteins are applied to other Omp fusion proteins, keeping in mind the structural configuration of the specific Omp-like protein.

For example, the native OmpA protein contains eight anti-parallel transmembrane β-strands within the 170 amino acid N-terminal domain of the protein. Between each pair of transmembrane domains is an extracellular or intracellular loop, depending on the direction of insertion of the transmembrane domain. The C-terminal domain consists of 155 amino acids which are located intracellularly and presumably contact the peptidoglycan occupying the periplasmic space. Expression vectors have been generated that facilitate the generation of OmpA fusion proteins. For example, Hobom *et al.*, 1995, Dev. Biol. Strand. 84:255-262 have developed vectors containing the OmpA open reading frame with linkers inserted within the sequences encoding the third or fourth extracellular loops that allow the in-frame insertion of the heterologous protein of choice.

In one embodiment of the invention, the portion of the OmpA fusion protein containing the gene product of interest has enhanced expression in the periplasm. In one aspect of the embodiment, the fusion protein comprises prior to maturation either the signal sequence or the signal sequence followed by at least one membrane-spanning domain of OmpA, located N-terminal to the gene product. The signal sequence is cleaved and absent from the mature protein. In another aspect of the embodiment, the gene product of interest is at the N-terminus of the OmpA fusion, rending inconsequential to the positioning of the gene product the number of membrane spanning domains of OmpA utilized, as long as the fusion protein is stable. In yet another aspect of the embodiment, the gene product is situated between the N - and C-terminal domains of OmpA such that a soluble periplasmic protein containing the gene product upon cleavage by a periplasmic protease within the periplasm. In all aspects of this embodiment, it is preferred that a bacterial vector which expresses a periplasmic gene product also co-express BRP to enhance release of the effector molecule from the bacterial cell, see *infra*.

In another embodiment of the invention, the portion of the OmpA fusion protein containing the gene product of interest is at the extracellular bacterial surface. In one aspect of the embodiment, the fusion protein comprises an even number or odd number of membrane-spanning domains of OmpA located N-terminal to the gene product. In another aspect of the embodiment, the gene product is situated between two extracellular loops of OmpA for presentation to the tumor cell by the bacterial cell. In specific

embodiments, the invention provides expression plasmids of gene product fusion proteins at the bacterial extracellular surface. For example, the plasmid denoted Trc(lpp)*ompA*, comprises a *trc* promoter-driven lipopolyprotein (lpp) anchor sequence fused to a truncated *ompA* transmembrane sequence. As another example, the plasmid is denoted Trc*ompA* comprises a *trc* promoter-driven *ompA* gene signal sequence. Such plasmids may be constructed to comprise a nucleic acid comprising or encoding one or more gene product(s) of the invention.

Optionally, a gene product of interest is preceded or flanked by consensus cleavage sites for a metalloprotease or serine protease that is abundant in tumors, for release of the gene product into the tumor environment. Whether the gene product of interest is preceded or flanked by protease cleavage sites depends on whether it is located terminally or internally in the fusion protein, respectively.

Similar fusion proteins may be constructed with any of the Omp-like proteins using the strategies described above in terms of OmpA. In the construction of such fusion proteins, as will be apparent to one of ordinary skill in the art, the selection of the portion of the Omp-like protein to be fused to a gene product of interest will depend upon the location that is desired for the expression of the gene product (e.g. periplasmic, extracellular, membrane bound, *etc.*).

Construction of fusion proteins for expression in bacteria are well known in the art and such methods are within the scope of the invention. See, *e.g.*, Makrides, S., 1996, Microbiol. Revs 60:512-538 which is incorporated herein by reference in its entirety.

In certain other embodiments of the invention, the attenuated tumor-targeted bacterial vectors of the invention, which express at least one gene product of interest, also express at least one secondary effector molecule which functions to permeabilize the bacteria cell membrane(s) or enhance the release of intracellular components into the extracellular environment, *e.g.* at the tumor site, thereby enhancing the delivery of the gene product of interest(s). Such a secondary effector molecule(s) which permeabilizes the bacterial cell or enhances release is designated "a release factor". In certain embodiments, the release factor also advantageously has anti-tumor activity.

The release factor expressed by the bacterial vector of the invention may be endogenous to the modified attenuated tumor-targeted bacteria or it may exogenous (*e.g.*, encoded by a nucleic acid that is not native to the attenuated tumor-targeted bacteria). A release factor may be encoded by a nucleic acid comprising a plasmid, or by a nucleic acid which is integrated into the genome of the attenuated tumor-targeted bacteria. A release factor may be encoded by the same nucleic acid or plasmid that encodes a primary effector molecule, or by a separate nucleic acid or plasmid. A release factor may be encoded by the

same nucleic acid or plasmid that encodes a secondary effector molecule, or by a separate nucleic acid or plasmid. In one embodiment, the release factor is expressed in a cell which also expresses a fusion protein comprising a primary effector molecule fused to an Omp-like protein. In this embodiment, the co-expression of the release factor allows for enhanced release of the fusion protein from the periplasmic space.

In a preferred embodiment, such a factor is one of the Bacteriocin Release Proteins, or BRPs (herein referred to in the generic as BRP). The BRP employed in the invention can originate from any source known in the art including but not limited to the cloacin D13 plasmid, one of colicin E1-E9 plasmids, or from colicin A, N or D plasmids. In a preferred embodiment, the BRP is of cloacin D13 (pCloDF13 BRP).

Generally, BRPs are 45-52 amino acid peptides that are initially synthesized as precursor molecules (PreBRP) with signal sequences that are not cleaved by signal endopeptidases. BRP activity is thought to be mediated, at least in part, by the detergent-resistant outer membrane phopholipase A (PldA) and is usually associated with an increase in the degradation of outer membrane phospholipid (for a general review on BRPs, see van der Wal *et al.*, 1995, FEMS Microbiology Review 17:381-399). Without limitation as to mechanism, BRP promotes the preferential release of periplasmic components, although the release of cytoplasmic components is also detected to a lesser extent. When moderately overexpressed, BRP may cause the bacterial membrane to become fragile, inducing quasi-lysis and high release of cytoplasmic components. Additionally, it is thought that when BRP is expressed at superhigh levels, the protein can cause bacterial cell lysis, thus delivering cellular contents by lytic release. In this embodiment, BRP expression may be correlated with BRP activity (*e.g.*, release of bacterial contents). For example, superhigh BRP activity results in bacterial cell lysis of substantially all bacteria. Thus, as used herein, superhigh expression is defined as the expression level of BRP which results in bacterial cell lysis of substantially all bacteria. Moderate BRP activity, is associated with partial or enhanced release of bacterial contents as compared to a control bacteria which is not expressing BRP, without obligate lysis of the bacteria. Thus, in this embodiment, moderate overexpression of BRP is defined as the expression level at which release of cytoplasmic components is enhanced, without bacterial lysis of substantially all of the bacteria. Substantially all of the bacteria, as used herein, is more than 60% of the bacteria, preferably more than 70%, more preferably 80%, still more preferably more than 90% and most preferably 90-100% of bacteria.

In a specific embodiment of the invention, the BRP protein is a pCloDF13 BRP mutant whose lytic function has been uncoupled from its protein release function, thereby enhancing protein release without bacterial lysis (van der Wal *et al.*, 1998, App.

Env. Microbiol. 64:392-398). This embodiment allows for prolonged protein release from the bacterial vector, while reducing the need for frequent administration of the vector. In another specific embodiment, the BRP of the invention is a pCloDF13 BRP with a shortened C-terminus, which in addition to protein release causes cell lysis (Luirink *et al.*, 1989, J. Bacteriol. 171:2673-2679).

In another embodiment of the invention, the enhanced release system comprises overexpression of a porin protein; *see e.g.*, Sugawara, E. and Nikaido, H., 1992, J. Biol. Chem.267:2507-11.

In certain embodiments when a BRP is expressed by the bacterial vector of the invention, the BRP may be endogenous to the modified attenuated tumor-targeted bacteria or it may be exogenous (*e.g.*, encoded by a nucleic acid that is not native to the attenuated tumor-targeted bacteria). A BRP may be encoded by a nucleic acid comprising a plasmid, or by a nucleic acid which is integrated into the genome of the attenuated tumor-targeted bacteria. A BRP may be encoded by the same nucleic acid or plasmid that encodes a primary effector molecule, or by a separate nucleic acid or plasmid. A BRP may be encoded by the same nucleic acid or plasmid that encodes a secondary effector molecule, or by a separate nucleic acid or plasmid. In one embodiment, the BRP-like protein is expressed in a cell which also expresses a fusion protein comprising a gene product of interest fused to an Omp-like protein. In this embodiment, the co-expression of the BRP allows for enhanced release of the fusion protein.

In a preferred specific embodiment of the invention a BRP encoding nucleic acid is encoded by a colicin plasmid. In another specific embodiment of the invention, the BRP encoding nucleic acid is expressed under the control of the native BRP promoter, which is an SOS promoter that responds to stress (*e.g.* conditions that lead to DNA damage such as UV light, X-irradiation) in its normal host (for BRP, *Enterococcus cloacae*), yet is partially constitutive in *Salmonella*. In a preferred embodiment, the BRP encoding nucleic acid is expressed under the control of the pepT promoter, which is activated in response to the anaerobic nature of the tumor environment (*see e.g.*, Lombardo *et al.*, 1997, J. Bacterio. 179:1909-17).

Alternatively, the promoter can be an antibiotic-induced promoter, such as the *tet* promoter of the Tn10 transposon. In a preferred embodiment, the *tet* promoter is a singlemer, which singlemer responds in an all-or-nothing manner to the presence of tetracycline and provides a genetically stable on-off switch. In another embodiment, the *tet* promoter is multimerized, for example three-fold. Such a multimer responds in a graded manner to the presence of tetracycline and provides a more manipulable system for control of effector molecule levels. Promoter activity would then be induced by administering to a

subject who has been treated with the attenuated tumor-targeted bacteria of the invention an appropriate dose of tetracycline. Although the *tet* inducible expression system was initially described for eukaryotic systems such as *Schizosaccharomyces pombe* (Faryar and Gatz, 1992, Current Genetics 21:345-349) and mammalian cells (Lang and Feingold, 1996, Gene 168:169-171), recent studies extend its applicability to bacterial cells. For example, Stieger *et al.*,1999, Gene 226:243-252 have shown 80-fold induction of the firefly luciferase gene upon *tet* induction when operably linked to the *tet* promoter. An advantage of this promoter is that it is induced at very low levels of tetracycline, approximately 1/100th of the dosage required for antibiotic activity.

Also, the promoter can be an SOS promoter that responds to stress (*e.g.* conditions that lead to DNA damage such as UV light, X-irradiation). In this manner the expression of the gene product of interest is induced upon exposure of the bacteria to a DNA damaging agent, *e.g.*, ionizing radiation. In a more preferred embodiment, the bacteria are genetically engineered to be more responsive to DNA damage by having a mutation at a locus involved in DNA repair or recombination or at a locus involved in the prevention of DNA damage due to oxygen free radicals, such as the *recN* or *oxyR* locus. Not intending to be limited to a particular mechanism, it is believed that upon exposure of such mutated bacteria, *e.g.*, *recN*$^-$ or *oxyR*$^-$ bacteria, to a DNA damaging agent such as X-irradiation, more single-stranded DNA is produced in the cell, which in turn activates the SOS promoter at a higher level compared to bacteria without the mutation. Brena-Valle et al., 1998, Mutagenesis 13:637-641. In a yet more preferred embodiment, the promoter has especially high sensitivity to SOS induction signals, *e.g.*, single-stranded DNA. Exemplary highly responsive promoters include the *sulA*, *recA*, and *recN* promoters.

5.2 IRRADIATION TREATMENT

The source of the irradiation can be gamma rays or X-rays. The treatment may comprise a single dose of irradiation or may comprise several doses of irradiation (fractionated doses). The effective dose of irradiation can be calculated using methods known in the art taking into account the overall health of the patient and the type and location of the solid tumor. An illustrative example of a course of radiation treatment for a human patient with a solid tumor is local administration of irradiation to the tumor site of 2 Gy/day for 5 days per week for 6 weeks (total exposure of 60 Gy). For a general overview of radiation therapy, see Hellman, Chapter 12: Principles of Radiation Therapy Cancer, in: Principles and Practice of Oncology, DeVita *et al.*, eds., 2nd. Ed., J.B. Lippencott Company, Philadelphia.

5.3 METHODS AND COMPOSITIONS FOR TREATMENT

According to the present invention, the tumor-targeted, bacteria or bacterial vectors which express a gene product of interest are advantageously used in a combination method with one or more doses of irradiation to produce a tumor growth inhibitory response or a reduction of tumor volume, in a subject, including a human patient, having a solid tumor cancer. In one embodiment of the present invention, the method comprises administering, to a subject, a pharmaceutical composition comprising an effective amount of facultative aerobic or facultative anaerobic tumor-targeted bacteria in combination with one or more doses of irradiation. In another embodiment of the present invention, the method comprises administering, to a subject, a pharmaceutical composition comprising an effective amount of facultative aerobic or facultative anaerobic, attenuated, tumor-targeted bacteria in combination with one or more doses of irradiation. In yet another embodiment, the method comprises administering, to a subject, a pharmaceutical composition comprising an effective amount of a facultative aerobic or facultative anaerobic, attenuated, tumor-targeted bacterial vector which has been genetically modified to express a gene product of interest, which gene product aids in reducing the volume, or inhibiting the growth of the tumor in combination with one or more doses or irradiation. In a preferred embodiment, the method comprises administering, to a subject, a pharmaceutical composition comprising an effective amount of a facultative aerobic or facultative anaerobic, attenuated, tumor-targeted *Salmonella spp.* which expresses a gene product of interest which aids in reducing the volume or inhibiting the growth of a solid tumor under the control of an irradiation-inducible promoter.

Solid tumors include, but are not limited to, sarcomas, carcinomas and other solid tumor cancers, including, but not limited to germ line tumors, tumors of the central nervous system, breast cancer, prostate cancer, bladder cancer, renal cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, mesoendothelioma, mesothelioma, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma. The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human. Effective treatment of a solid tumor, includes but is not limited to, inhibiting tumor growth, reducing tumor volume, etc..

The amount of the pharmaceutical composition of the invention which is effective in the treatment of a solid tumor cancer will depend on the nature of the solid tumor, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed will also depend on the route of administration, and the seriousness of the solid

tumor, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges are generally from about 1.0 c.f.u./kg to about 1 x 10^{10} c.f.u./kg; optionally from about 1.0 c.f.u./kg to about 1 x 10^{8} c.f.u./kg; optionally from about 1 x 10^{2} c.f.u./kg to about 1 x 10^{8} c.f.u./kg; optionally from about 1 x 10^{4} c.f.u./kg to about 1 x 10^{8} c.f.u./kg. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The amount and frequency of irradiation which will be effective in the treatment of a solid tumor cancer will depend on the nature and location of the solid tumor, and can be determined by standard clinical techniques. The precise dose to be employed will also depend on the seriousness of the solid tumor, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable total dosage ranges are generally from about 1 Gray (Gy) to about 75 Grays; optionally from about 5 Gy to about 25 Gy; optionally from about 10 Gy to about 15 Gy.

Various delivery systems are known and can be used to administer a pharmaceutical composition of the present invention. Methods of introduction include but are not limited to intradermal, intrathecal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the bacteria or bacterial vector can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald *et al.*, Surgery 88:507

(1980); Saudek *et al.*, N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); *see also* Levy *et al.*, Science 228:190 (1985); During *et al.*, Ann. Neurol. 25:351 (1989); Howard *et al.*, J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration

to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The following series of examples are presented by way of illustration and not by way of limitation on the scope of the invention.

6. EXAMPLES

The following experiments demonstrate the anti-tumor efficacy of attenuated strains of *Salmonella* alone, and in combination with irradiation treatment (X-rays) against murine and human tumor models in mice. As the results illustrate, each treatment alone slowed tumor growth and prolonged survival, but the combined treatments produced supra-additive, *i.e.*, enhanced anti-tumor effects, *i.e.*, more than the additive effect expected following administration of tumor-targeted bacteria and irradiation together. In other words, in dose response studies with a single dose of *Salmonella* and increasing doses of X-rays, the two agents together caused suppression of tumor growth that was greater than that calculated for additive effects of the two agents. The experiments also demonstrate that the greatest anti-tumor effect was observed with an attenuated strain of *Salmonella* expressing a ribonuclease, *i.e.*, colicin E3, in combination with X-irradiation.

6.1. MATERIALS AND METHODS

Bacterial and cancer cells: *msbB⁻, purI⁻ Salmonella* strains YS1646, ATCC Accession No. 202165, designated VNP200009 herein and YS1456 (ATCC Accession No. 202164) were used and are described in International Publication WO 99/13053, which is incorporated by reference in its entirety. B16F10 mouse melanoma cells were kindly provided by Dr. I. Fidler, M.D. (Anderson Cancer Center, Houston, TX), EMT-6 mouse mammary cells were provided by Dr. Sara Rockwell (Yale University School of Medicine, New Haven, CT), and DLD-1 human colon carcinoma cells were obtained from the American Type Culture Collection. B16F10 and DLD-1 cells were maintained in DMEM medium with 10% horse or bovine sera and antibiotics by routine culture methods. EMT-6

cells were maintained in Waymouth's medium with 15% fetal bovine serum, and antibiotics.

Tumor implantation and bacterial injection: Tumors were implanted on the mid-right side of the mice. Female C57B6 mice 6-8 weeks old were implanted subcutaneously (s.c.) with 5 x 10^5 B16F10 cells; female BALBc *nulnu* mice 6-8weeks old were implanted s.c. with 10^7 DLD-1 cells; and male BALB/c RW mice 8-10 weeks old were implanted intradermally (i.d.) with 2 x 10^5 EMT-6 cells. Where indicated, mice were further inoculated i.p. or i.v. with one of the attenuated strains of *Salmonella*, at 2 x 10^5 cfu/mouse unless otherwise noted, in 0.2ml 0.9% saline, at periods from 7 to 14 days post-tumor implantation as noted.

X-irradiation: X-rays were administered using a Siemens Stabilipan 250 kV 15mA X-ray machine with a 2mm aluminum filter. The dose rate was 1.109 Gy/min.

Tumor growth: Mice were tagged and followed individually over time for tumor growth. Tumor measurements were collected with Mitutoyo Digimatic Calipers connected to an Apple PowerBook® computer. Volume was calculated and data were rearranged with the Scriptable Text Editor and AppleScript™ software from Apple Computer, Inc. The formula: volume = L x W x H x 0.52 was used for volume calculations. Animals were euthanized when tumors reached 4000 mm^3, or when the animals became moribund. Effects of treatments on tumor growth were expressed either as mean tumor volume versus time, the time (days) to attain 1 g tumors, or as the percent suppression of tumor growth expressed as 1-T/C where T is treated volume and C is control tumor volume. For example, if treated tumors were 20% of the volume of control tumors on a given day, tumor suppression in the treated group was 80% (see, *e.g.*, Table 1). In one experiment (FIG. 3), DLD-1 tumor growth was expressed as percent Initial Volume because the mean tumor volumes between different treatment groups differed by more than two-fold on the day of treatment.

Statistics: Means and standard errors were calculated according to standard formulas with AppleScript™ software. Curve fitting was done with Cricket Graph III® from Computer Associates, Inc. Other statistics, including p-value calculations, were done with StatView® from Abacus Concepts, Inc.

Antibiotics *in vivo*: In experiments with attenuated *Salmonella* strain YS1456, 2weeks following injection of bacteria, mice were given BAYTRIL™, enrofloxacin, a quinolone antibiotic (Bayer, Inc) diluted 1:100 in the drinking water for 1 week every other week for up to 7 weeks post tumor implantation. No antibiotics were used with *Salmonella* strain VNP200009.

6.2. RESULTS

6.2.1. *SALMONELLA* AND TUMOR GROWTH SUPPRESSION

C57B6 mice were implanted with 5 x 10^5 B16F10 melanoma cells. *Salmonella* strain VNP20009 was injected i.p. 7 days later at the c.f.u. indicated in Table 1. Tumor growth was assessed after 20 days as described in Section 6.1. The percent suppression of tumor growth was calculated as described in Section 6.1. Results are presented in Table 1. Results represent mean for n=10 mice per group.

Salmonella suppressed the growth of B16F10 melanomas as shown in Table 1. The number of bacteria inoculated had only a small effect on tumor suppression, as shown for i.p. injections of 2 x 10^5 and 2 x 10^6 colony forming units (c.f.u.), which inhibited tumor growth by 78% and 80% respectively, 20 days post tumor implantation (Table 1).

Table 1

***Salmonella* and tumor growth suppression in C57B6 mice bearing B16F10 melanomas.**

Strain	c.f.u. injected	% tumor suppression
VNP20009		
	none	-0-
	2 x 10^5	78%
	2 x 10^6	80%
YS1456		
	none	-0-
	2 x 10^5	84%
	2 x 10^6	88%

In a similar experiment with i.v. injections, bacterial inoculi were increased in log increments over a 100-fold range, while tumor suppression increased from 80% (10^4cfu/mouse) to 94% (10^6cfu/mouse) (data not shown). This lack of strong dependence of tumor suppression on the number of inoculated bacteria results from amplification of the *Salmonella* within tumors (Pawelek *et al.*, 1997, Cancer Res. 57:4537-4544; Low *et al.*, 1999, Nature Biotechnology 17:37-41). For example, when *Salmonella* were injected i.p. into B16F10 melanoma-bearing mice in log doses from 10^2 to 10^5 c.f.u./mouse, tumor bacterial counts consistently reached levels of 10^9 c.f.u./g by 6 days (not shown).

Therefore, with *Salmonella*, the final numbers of bacteria eventually plateau at similar levels within tumors, independent of the original bacterial inoculum. Thus, in the experiments below, single doses of *Salmonella*/mouse were used throughout in combination with both single and multiple doses of X-irradiation.

6.2.2 *SALMONELLA* AND SINGLE X-IRRADIATION DOSE

The effects of single X-irradiation dose from 5 to 15 Gy, with and without i.v. injected *Salmonella* strain VNP20009 on B16F10 melanoma growth suppression in mice were investigated. Anti-tumor activity was assessed as the number of days to attain 1 gram weight tumors. Results are presented in FIG.1. *Salmonella* strain VNP20009 alone prolonged the time to attain 1gram in weight from the control value of 18±1 days to a value of 26±3 days. The dose-response curve relating radiation dose to tumor growth delay was linear over the dose range studied. X-rays alone also prolonged the time to attain 1 gram tumor weight. The combination of *Salmonella* and X-irradiation showed unexpected supra-additive anti-tumor effects, with the slope of the dose-response curve being greater than expected for additivity following administration of tumor-targeted bacteria and irradiation together (FIG. 1). This supra-additivity was observed in all three of the three X-ray dose-response experiments in mice using the B16F10 melanoma tumor model, as shown by the actual slopes of the dose-response curves obtained versus the slopes expected for simple additivity, (see Table 2).

Table 2

Treatment	Expt #	Slope + y-intercept*	r^2
X-irradiation only (0-15 Gy)	177	y=0.605x + 18.0	0.99
	172	y=0.490x + 17.9	0.60
	185	y=0.524x + 16.4	0.99
	pooled data	y=0.514x + 17.6	0.83
Salmonella and X-irradiation	177	y=1.089x + 26.6	0.99

(0-15 Gy)	172	y=1.350x + 21.2	0.99
	185	y=0.912x + 21.5	0.43
	pooled data	y=1.075x + 23.3	0.61
expected for additivity	177	y=0.605x + 26.7	n.a.
	172	y=0.490x + 21.2	n.a.
	185	y=0.524x + 21.5	n.a.
	pooled data	y=0.514x + 23.3	n.a.

*y intercept for X-rays only = d to 1g for sham X-rayed controls (c.f. Fig 1); y intercept for *Salmonella* + X-rays = d to 1g with *Salmonella* only + sham radiation. r^2 = correlation coefficient.

Tumor growth curves from the experiment described in Section 6.2.1 are shown in FIG. 2 where it is seen that the combination of strain VNP20009 and a singe dose of X- irradiation at 15 Gy markedly slowed B16F10 melanoma growth and prolonged mouse survival compared to either bacterial or irradiation treatment alone.

In another series of experiments, similar results were obtained using the slower-growing DLD-1 human colon carcinoma xenografted into *nu/nu* mice. BALBc *nu/nu* mice were implanted s.c. with 10^7 DLD-1 cells. At 13 days post-implantation, when tumors were about 0.5 gram, the mice were separated into 4 groups, and two of the groups were injected i.v. with 8 x 10^5 c.f.u. *Salmonella* strain VNP20009. At 17 days post-implantation, one group injected with the *Salmonella* and one group not injected were irradiated with 15 Gy X-ray irradiation; a fourth group served as a non-treated control group. The results are presented in FIG. 3. Again, the anti-tumor effects of strain VNP20009 with a single dose of X-irradiation at 15 Gy were greater than the effects seen for treatments either with the *Salmonella* strain or with X-irradiation alone.

6.2.3 *SALMONELLA* AND MULTIPLE X-IRRADIATION DOSES

Multiple (fractionated) X-ray dosing experiments were carried out with cumulative total irradiation of 25 Gy and 50 Gy. In a representative experiment with 25 Gy, when B16F10 melanomas had reached about 0.5 gram (14 days post-implantation), animals were either treated with 10 Gy X-irradiation alone, with 10 Gy irradiation and i.v. administration of 2 x 10^5 c.f.u. *Salmonella* strain YS1456, i.v. administration with 2 x 10^5

c.f.u. *Salmonella* strain YS1456 alone, or received no treatment (control). Each of the groups aside from the control group subsequently received three weekly doses of X-irradiation at 5 Gy per dose. Antibiotic treatment with BAYTRIL™ was employed throughout the experiment as described in Section 6.1. The results are shown in FIG. 4.

The results shown in FIG. 4 demonstrate that although tumor growth was suppressed by both *Salmonella* and irradiation alone, the combined treatment showed significant increases in both tumor suppression and survival over the individual treatments alone. Since dose-response studies were not carried out with the fractionated X-ray protocols, it could not be determined rigorously whether treatment with the combination of *Salmonella* and X-irradiation produced additive or supra-additive effects.

In another series of experiments, similar results for multiple X-ray treatments with a cumulative total of 25 Gy were obtained with EMT-6 mouse mammary tumors implanted i.d. in BALB/c mice. Briefly, 8 to 10 week old male BALBc mice were injected i.d. with 2 x 10^5 EMT-6 cells. When tumors reached about 0.5 gram weight, the mice were separated into four groups, one group received no treatment (control), another group received local irradiation at 10 Gy alone, a third group received i.p. injections of 2 x 10^5 c.f.u. *Salmonella* strain YS1456 alone, and a fourth group received local irradiation at 10 Gy and i.p. injection of 2 x 10^5 c.f.u. *Salmonella* strain YS1456. Thereafter, all the groups except the control group received a weekly X-irradiation dose of 5 Gy for 3 weeks. Antibiotic treatment with BAYTRIL™ was terminated 32 days post-implantation. The results are presented in FIG. 5. As with the B16F10 melanomas described above, growth of EMT-6 tumors was suppressed by either *Salmonella* or X-ray treatment alone, but the strongest suppression was achieved with the combined treatments. As above, X-ray dose response studies were not carried out for this protocol.

In yet another series of experiments, it was observed that the strongest tumor suppression and longest survival of mice were achieved at the highest cumulative doses of X-irradiation (50 Gy) with *Salmonella* treatment. In these experiments, 5 x 10^5 B16F10 cells were implanted s.c. and at 7 days post-implantation of the B16F10 melanoma cells (when tumors were still not palpable), the mice were separated into four groups, two groups of which received i.v. administration of 2 x 10^5 c.f.u. *Salmonella* strain Y51456. Two days following administration of the bacteria, in one group injected with bacteria and in one group not injected with bacteria, the implantation sites were locally irradiated with 20 Gy X-irradiation. All but the control group then received weekly irradiation treatment at 10 Gy for 3 weeks. Antibiotic treatment with BAYTRIL™ was terminated 49 days post-implantation. The results are presented in FIG.6. For the combination of X-irradiation and *Salmonella* treatment, B16F10 melanomas attained a weight of 1 g on average 100 days

post-implantation, which is six times longer than the control group, five times longer than with *Salmonella* treatment alone, and about 50% longer than with X-irradiation treatment alone.

6.2.4 *SALMONELLA*-EXPRESSING TOXIN AND X-IRRADIATION

The following experiment demonstrates the anti-tumor ability of multiple X-irradiation doses alone, administration of *Salmonella* strain VNP2009 alone, administration of *Salmonella* strain VNP200009/E3 alone, which strain contains the plasmid Col E3-CA38 (GenBank Accession No. AF 129270) and expresses a ribonuclease under the control of a radiation-inducible promoter, and in combination with multiple doses of X-irradiation and either of the bacterial stains.

5-7 week old C57B6 female mice were implanted s.c. with 5 x 10^5 B16F10 mouse melanoma cells. 7 days post-implantation the mice were divided into the treatment groups set forth in Table 3. Bacteria were administered 10^5 c.f.u.; local X-irradiation was administered at 15 Gy at 12 and 26 days post-implantation ("dpt"). Tumors were measured twice per week with electronic calipers, and tumor volumes were calculated as described in Section 6.1. The results are shown in Table 3. (T/C is treatment divided by control).

Table 3

Category	mice/group n=( )	days to 1 gram (indiv. mice)	days to 1g mean	T/C
control	(6)	12,12,18,18,18,21	17	1.0
15 Gy x-rays 12dpt, 26dpt	(9)	14,14,18,21,25,35,35,67,67	33	1.9
sham +*Salmonella*	(8)	21,21,25,25,25,28,28,39 VNP20009 (10^5 c.f.u.)	27	1.6
sham +*Salmonella*	(8)	14,18,18,18,18,18,28,28 VNP20009/E3 (10^5 c.f.u.)	20	1.2

15 Gy x-rays 12dpt, 26dpt +*Salmonella* VNP20009 (10^5 c.f.u.)	(9)	21,28,35,35,56,60,60,60,67	47	2.8
15 Gy x-rays 12dpt, 26dpt +*Salmonella* VNP20009/E3 (10^5 c.f.u.)	(9)	28,39,53,56,60,67,74,78	57	3.3

As shown in Table 3, treatment of melanoma-bearing mice with *Salmonella* VNP20009/E3 in combination with X-irradiation (15 Gy weekly for two weeks; 30 Gy total) produced a significant delay in the onset of tumor growth compared to all other treatment categories, including VNP20009 without expressing the ribonuclease encoded by the Col E3-CA38 plasmid.

6.3 DISCUSSION

The above results demonstrate anti-tumor effects in three tumor models by combined administration of lipid A-modified, tumor-targeted *Salmonella* and X-rays. With all three tumor models, when treatment was initiated after tumors were palpable, numerous cases of tumor regression were documented in individual experimental animals, most frequently with the combined treatments of *Salmonella* and X-irradiation. Moreover, when the combination treatment, *i.e.*, *Salmonella* and X-irradiation, was given, there was a statistically significant inhibition of tumor growth (compared to control).

The combination of irradiation and *Salmonella* was therapeutically beneficial with radiation protocols of single and fractionated X-ray treatments, and a range of cumulative X-rays doses from 5-50 Gy. Bacterial protocols involved two different strains of *Salmonella* that were injected either i.p. or i.v. *Salmonella* injection times varied from several days prior, to immediately following, irradiation. Although, not intending to be limited to a particular mechanism of action, the finding that the *Salmonella* and X-ray combination was beneficial even when the bacteria were injected after irradiation indicates that the bacteria were not acting as classic radiosensitizers (Steel and Peckham, 1979, Int. J. Radiation Oncology Biol. Phys. 5:85-91).

In experiments not shown herein, it was found that exposure of the *Salmonella* cultures to 2O Gy X-rays *in vitro,* the largest single dose used herein in the tumor/animal models, resulted in >90% survival of the bacteria. Thus, the X-rays used in the tumor experiments above would have had very little effect on survival of *Salmonella* in the tumors.

We use the term *supra-additivity*, as discussed by Steel and Peckham, 1979, Int. J. Radiation Oncology Biol. Phys. 5:85-91 for our combined radiotherapy and bacterial therapy in which the administration of one agent apparently increases the effect of another, or in which the effect of a combination appears to be greater than would be expected for either agent alone. The term *synergism* was not used because the term implies that the two agents are working together, and as stated above, the mechanisms underlying the supra-additive anti-tumor effects of *Salmonella* and X-rays are unknown. In any event, the results demonstrate that the combined use of *Salmonella* and X-rays are of therapeutic value in cases where X-irradiation is indicated for the treatment of human cancer.

7. MICROORGANISM DEPOSITS

The following microorganisms were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, on 25 August, 1998, and have been assigned the indicated Accession numbers:

Microorganism	ATCC Accession No.
YS1646	202165
YS1456	202164

The invention claimed and described herein is not to be limited in scope by the specific embodiments, including but not limited to the deposited microorganism embodiments, herein disclosed since these embodiments are intended as illustrations of several aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.

We claim:

1. A method for reducing the volume of or inhibiting the growth of a solid tumor comprising administering an effective amount of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria in combination with at least one dose of irradiation, to a patient having a solid tumor cancer for reduction of tumor volume or inhibition of tumor growth.

2. The method according to claim 1 wherein the isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is an isolated population of attenuated, tumor-targeted bacteria.

3. The method according to claim 2 wherein the attenuated, tumor-targeted bacteria population is a super-infective population.

4. The method according to claim 2 wherein the attenuated population induces TNFα expression from about 1 to about 75 percent as compared to a corresponding non-attenuated population.

5. The method according to claim 1 wherein the isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is selected from the group consisting of *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis*.

6. The method according to claim 5 wherein the bacteria are *Salmonella spp.*

7. The method according to claim 3 wherein the isolated population of attenuated, super-infective, tumor-targeted bacteria is produced by:

(a) exposing a cell culture of a solid tumor cancer to an attenuated bacterium for a time sufficient so that the attenuated bacterium can infect the tumor cells; and

(b) isolating a population of attenuated, super-infective, tumor-targeted bacteria from the infected cell culture.

8. The method according to claim 1 wherein the isolated population of tumor-targeted bacteria is produced by:

(a) exposing a bacterium to tumor cell conditioned medium for a time sufficient to allow the bacterium to chemotact towards the tumor cell conditioned medium; and

(b) isolating a population of bacteria which chemotact towards the tumor cell conditioned medium.

9. The method according to claim 3 wherein the isolated population of attenuated, super-infective, tumor-targeted bacteria is produced by:

(a) exposing a mammal having a solid tumor cell cancer to an attenuated bacterium for a time sufficient so that the attenuated bacterium can infect the tumor cells; and

(b) isolating a population of attenuated, super-infective, tumor-targeted bacteria from the infected tumor cells.

10. The method according to claim 1, 7, 8, or 9 wherein the solid tumor cancer is selected from the group consisting of breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, renal cancer, bladder cancer, mesothelioma and melanoma.

11. A method for reducing the volume of or inhibiting the growth of a solid tumor comprising administering an effective amount of a single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria, which clone replicates preferentially at a tumor site, in combination with at least one dose of irradiation, to a patient having a solid tumor cancer for reduction of tumor volume or inhibition of tumor growth.

12. The method according to claim 11 wherein the single colony clone is a single colony clone of an isolated population of attenuated, tumor-targeted bacteria.

13. The method according to claim 12 wherein the attenuated single colony clone induces TNFα expression from about 1 to about 75 percent as compared to a corresponding non-attenuated single colony clone.

14. The method according to claim 11 wherein the single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria is

selected from the group consisting of *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Streptococcus spp.*, *Yersinia enterocolitica*, *Listeria monocytogenies*, and *Mycoplasma hominis*.

15. The method according to claim 14 wherein the bacteria are *Salmonella spp.*

16. The method according to claim 11 wherein the single colony clone expresses a gene product of interest which product aids in reducing the volume of, or inhibiting the growth of, the solid tumor cancer.

17. The method according to claim 16 wherein the gene product of interest is selected from the group consisting of a cellular toxin, cytokine and an anti-angiogenic protein.

18. The method according to claim 16 wherein the gene product of interest is a colicin.

19. The method according to claim 1 or 11 wherein the irradiation dose is X-irradiation or gamma-irradiation.

20. The method according to claim 1 or 11 wherein the irradiation dose is administered after administration of the isolated population or single colony clone.

21. The method according to claim 1 or 11 wherein the irradiation dose is administered before administration of the isolated population or single colony clone.

22. The method according to claim 1 or 11 wherein effective amount is from about 1 to about $1x10^9$ c.f.u./kg.

23. The method according to claim 1 or 11 wherein the effective amount is from about $1x10^2$ to about $1x10^8$ c.f.u./kg.

24. The method according to claim 12 wherein the single colony clone of an isolated population of attenuated, tumor-targeted bacteria is a single colony clone of an isolated population of attenuated, tumor-targeted *recN*$^-$ or *oxyR*$^-$ bacteria.

25. The method according to claim 16 wherein the gene product of interest is expressed under the control of an inducible promoter.

26. The method according to claim 25 wherein the inducible promoter is an SOS promoter.

27. The method according to claim 26 wherein the SOS promoter is selected from the group of promoters consisting of *recA*, *sulA*, *umuC*, *dinA*, *dinB*, *dinD*, *lexA*, *ruv*, *uvrA*, *uvrB*, *uvrD*, *umuD*, *cea*, *caa*, and *recN*.

28. The method according to claim 26 wherein the SOS promoter is selected from the group of promoters consisting of *recN*, *recA* and *sulA*.

29. The method according to claim 26 wherein the SOS promoter is selected from the group of promoters consisting of *oxyR* and *soxR*.

30. A method for reducing the volume of or inhibiting the growth of a solid tumor comprising administering an effective amount of a single colony clone of an isolated population of facultative aerobic or facultative anaerobic tumor-targeted bacteria, which clone replicates preferentially at a tumor site and which clone is *recN*$^-$ or *oxyR*$^-$, in combination with at least one dose of irradiation localized to the tumor site, to a patient having a solid tumor cancer for reduction of tumor volume or inhibition of tumor growth.

31. A method for screening to identify bacteria that are more responsive to a DNA damaging agent comprising:

(a) exposing a bacterial culture containing a plasmid coding for the colicin E3 toxin under the control of a SOS-type promoter to a DNA damaging agent, wherein the bacterial culture is a culture of facultative aerobic or facultative anaerobic tumor-targeted bacteria;

(b) culturing the bacterial culture for a time sufficient for expression of the E3 toxin;

(c) diluting the bacterial culture by at least a factor of 100;

(d) plating the diluted bacterial culture on a bacterial culture plate containing a lawn of E3 toxin-sensitive bacteria;

(e) culturing the bacterial culture for a time sufficient for the E3 toxin to detectably kill the E3 toxin sensitive bacteria; and

(f) measuring a clearing zone around the bacterial culture plated on the culture plate,

wherein those bacterial cultures having larger clearing zones are more responsive to the DNA damaging agent.

32. The method according to claim 30 in which the bacterial culture is diluted by at least a factor of 1000.

33. The method according to claim 31 wherein the bacterial culture is subject to mutagenesis treatment and the size of the clearing zone is compared to a corresponding bacterial culture not subject to mutagenesis.

34. A method for reducing the volume of or inhibiting the growth of a solid tumor comprising administering an effective amount of a single colony clone of a bacterial culture identified by the method of claim 31, in combination with at least one dose of irradiation, to a patient having a solid tumor cancer for reduction of tumor volume or inhibition of tumor growth.

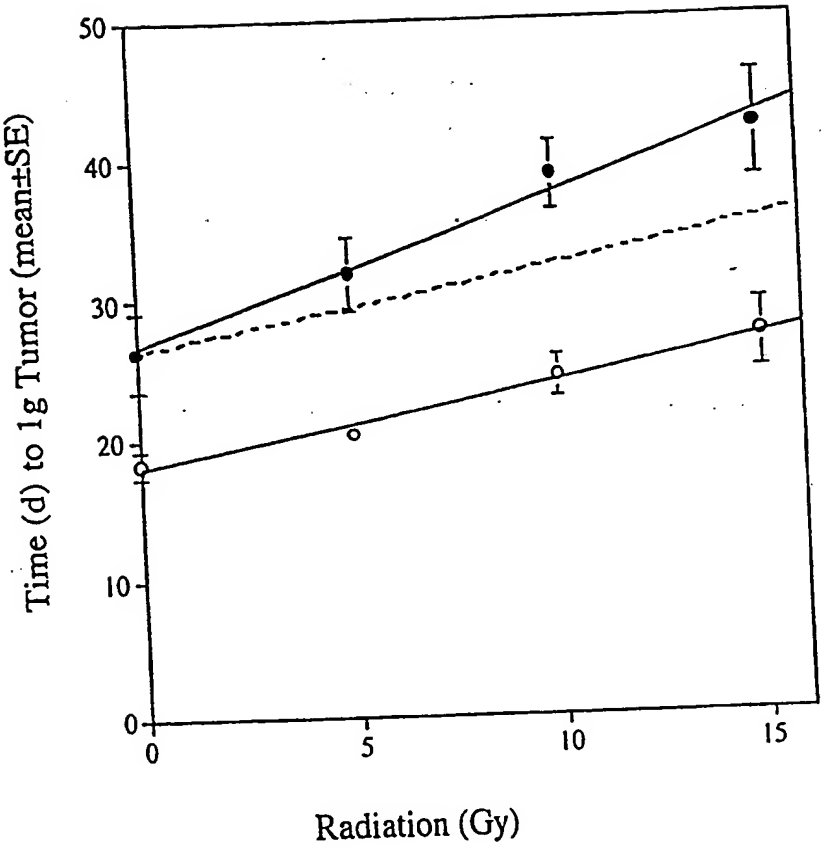


FIG. 1

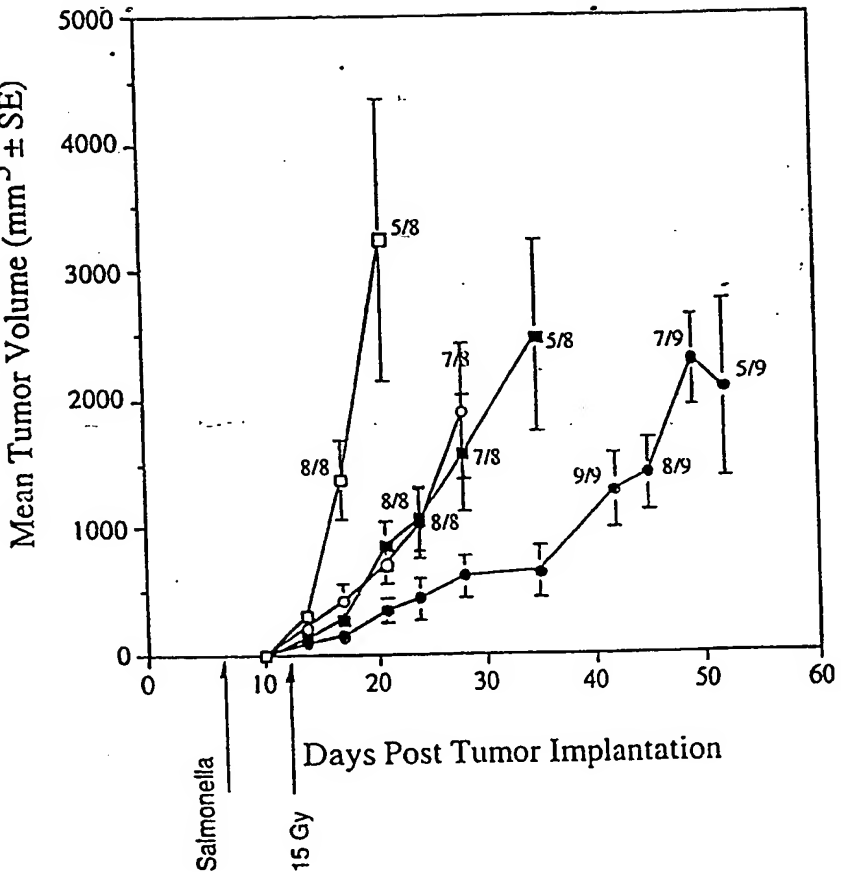

Mean Tumor Volume (mm^3 ± SE)
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
Days Post Tumor Implantation
Salmonella
15 Gy
5/8
8/8
8/8
8/8
7/8
7/8
5/8
9/9
8/9
7/9
5/9

FIG. 2

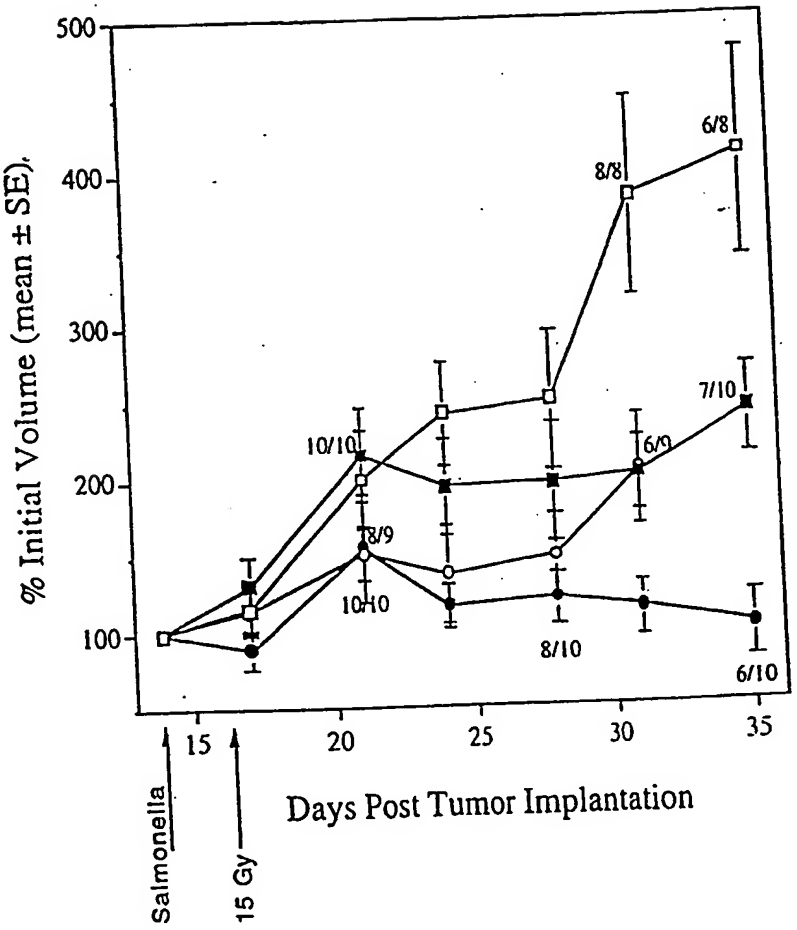

500
400
300
200
100
% Initial Volume (mean ± SE)
6/8
8/8
7/10
10/10
6/9
8/9
10/10
8/10
6/10
15
20
25
30
35
Salmonella
15 Gy
Days Post Tumor Implantation

FIG. 3

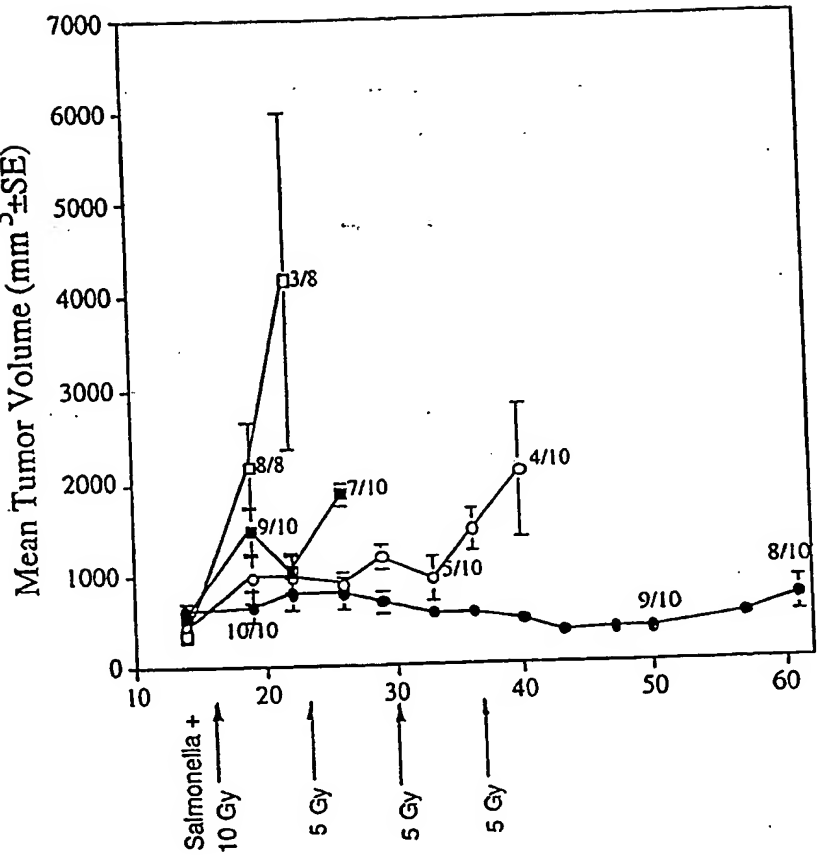

Mean Tumor Volume (mm^3±SE)
7000
6000
5000
4000
3000
2000
1000
0
10
20
30
40
50
60
3/8
8/8
7/10
9/10
4/10
5/10
8/10
9/10
10/10
Salmonella +
10 Gy
5 Gy
5 Gy
5 Gy
Days Post Tumor Implantation

FIG. 4

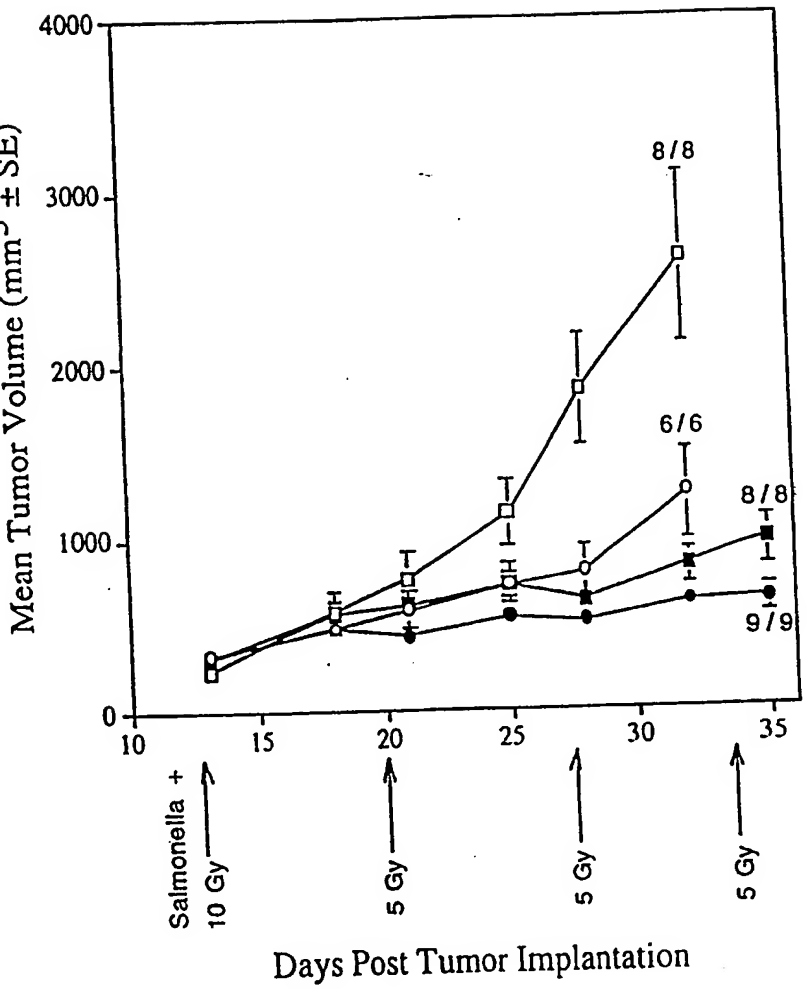

4000
3000
2000
1000
0
Mean Tumor Volume (mm3 ± SE)
8/8
6/6
8/8
9/9
10
15
20
25
30
35
Salmonella + 10 Gy
5 Gy
5 Gy
5 Gy
Days Post Tumor Implantation

FIG. 5

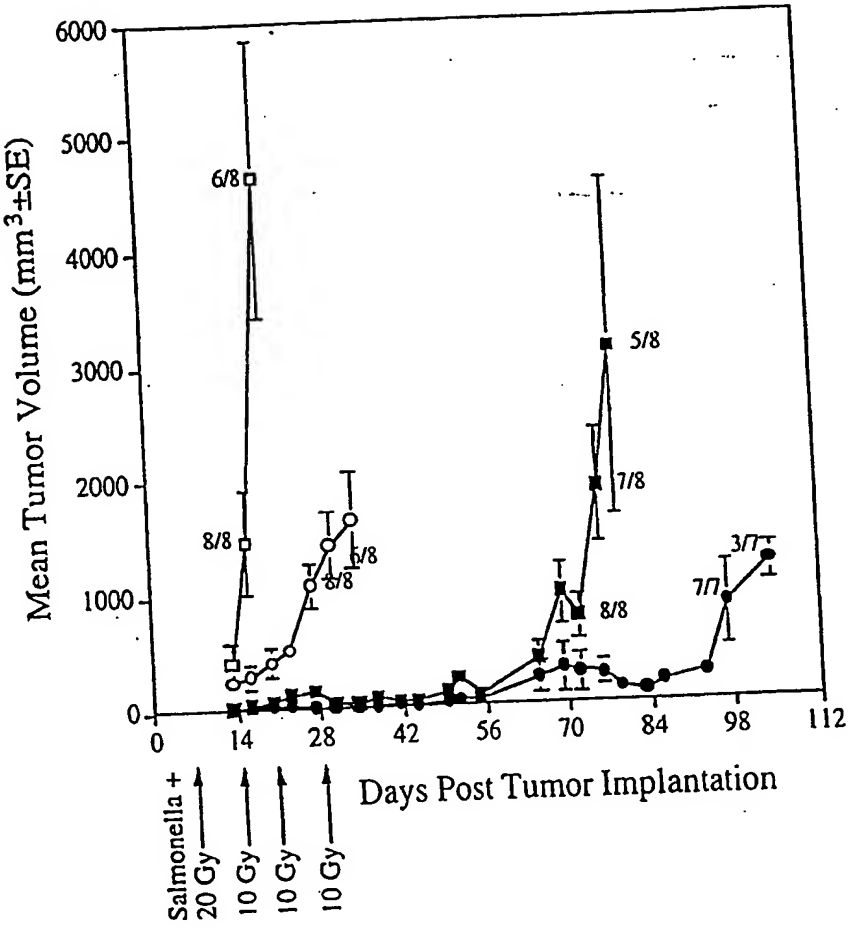

Mean Tumor Volume (mm3±SE)
6000
5000
4000
3000
2000
1000
0
6/8
8/8
6/8
6/8
5/8
7/8
8/8
7/7
3/7
0
14
28
42
56
70
84
98
112
Days Post Tumor Implantation
Salmonella +
20 Gy
10 Gy
10 Gy
10 Gy

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27391

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01N 63/00
US CL : 424/93.4, 93.44, 93.48
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.4, 93.44, 93.48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96/40238 A1 (YALE UNIVERSITY) 19 December 1996; the abstract; page 1, lines 10-20; page 13, line 19, through page 14, line 33; page 25, lines 1-16; page 26, Table 1; page 27, line 16, through page 32, line 14; and page 39, line 11, through page 40, line 11.	1-34
Y	COLLINS et al., 'Effects of Multiple Treatment with Corynebacterium parvum and Multifraction Tumor X-Irradiation on the Growth of Murine Tumors'. Radiation Research. June 1978, Vol. 74, No. 3, (New York), page 544, the abstract No. Eg-9,	1-34

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 DECEMBER 2000	Date of mailing of the international search report 28 FEB 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRENDA BRUMBACK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27391

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANDO et al. 'Intestinal Bacteria in Charge of Radiation-Induced Inhibition of Tumor Metastases'. Radiation Research Society. 25-29 March 1984, 32nd Annual Meeting, Orlando FL, page 132, the abstract No. Ji-23,	1-34
Y	KHODAREV et al. Role of Bacterial Pathogens in Host Cell Apoptosis: Involvement of Mycoplasmal Endonucleases in Nuclear Apoptopic Pathways and Sensitization of Tumor Cells to Irradiation and Drug-Induced Apoptosis. Proceedings of the American Association for Cancer Research. March 1999, Vol. 40, page 219, the abstract No. 1449,	5
Y	EISSNER et al. Critical Involvement of Transmembrane Tumor Necrosis Factor-α in Endothelial programmed Cell Death Mediated by Ionizing Radiation and Bacterial Endotoxin. Blood. December 1995, Vol. 86, No. 11, pages 4184-4193, especially the abstract.	4 and 13
Y	MULLEN et al. Treatment of Microscopic Pulmonary Metastases with Recombinant Autologous Tumor Vaccine Expressing Interleukin 6 and Escherichia coli Cytosine Deaminase Suidice Genes. Cancer Research. 15 March 1996, Vol. 56, pages 1361-1366, especially the abstract.	17
Y	FARKES-HIMSLEY et al. The Bacterial Colicin Active Against Tumor Cells In Vitro and In Vivo is Verotoxin 1. Proc. Natl. Acad. Sci., USA. July 1995, Vol. 92, pages 6996-7000, especially the abstract and first two paragraphs.	17 and 18
Y	BURES et al. Colicinogeny in Colorectal Cancer. Neoplasma. 1986, Vol. 33, No. 2, pages 233-237, especially the abstract.	17 and 18
Y	WO 97/41251 (VAN DER LELIE et al.) 06 November 1997, the abstract; page 1, lines 12-18; page 2, lines 20-21; page 3, line 5; page 7, lines 1-5 and 28-29; and page 13, lines 21-35.	24 and 30
Y	MASAKI et al. Colicin E3 and Its Immunity Genes. Journal of Molecular Biology. 1985, Vol. 182, pages 217-227, especially page 217, the abstract and first two paragraphs and page 218, first full paragraph.	26-28 and 31
Y	Database Pascal on Dialog, No. 08973064, TARTAGLIA et al. 'Identification and molecular analysis of OxyR-Regulated Promoters Important for the Bacterial Adaptation to Oxidative Stress', abstract, Journal of Molecular Biology, 1989, Vol. 210, No. 4, pages 709-79.	29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27591

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Database Pascal on Dialog, No. 12830981, DUKAN et al. 'Hypochlorous Acid Activates the Heat Shock and SoxRS Systems of Escherichia coli', abstract, Applied and Environmental Microbiology, 1996, Vol. 62, No. 11, pages 4003-4008.	29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27391

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG: Medline, BIOTECH, Conference Papers, PATENTS
search terms: tumor, tumour, carcinoma, sarcoma, treat?, therapy, therapeutic, salmonella, escherichia, E. coli, shigella, streptococcus, yersinia, listeria, mycoplasma, irradiat?, radiat?, attenuate?, colicin, promoter, sos, reca, recn, sula, oxyr, soxr, reca